Revisiting co-trimoxazole for the treatment of tuberculosis

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INTRODUCTION

INTRODUCTION

In 1882, *Mycobacterium tuberculosis* (*M. tuberculosis*) was identified as the cause of tuberculosis (TB) by Robert Koch. TB is an infectious disease that is transmitted from person to person: humans are the reservoir of *M. tuberculosis*. Transmission occurs by inhalation of aerosolized droplets loaded with viable bacilli expelled into the air through coughing and sneezing, by individuals with pulmonary TB. TB primarily affects the lungs, and indeed, most TB patients have pulmonary TB; but TB may also affect the central nervous system and virtually any other organ system. In 1905, Koch was awarded with the Nobel Prize for his discovery of the tubercle bacillus, but only in 1943, the first anti-TB drug – streptomycin was discovered, for which Selman Waksman was awarded with the Nobel Prize in 1952. In affluent countries, TB incidence had already started to decline well before the advent of effective antimicrobial chemotherapy in the 1950s. Philip D'Arcy-Hart working with the Tuberculosis Unit of the British Medical Research Council conducted the first randomized controlled trial showing a survival advantage for individuals given streptomycin [1]. In this trial it was shown that mono-therapy with streptomycin resulted in the emergence of drug-resistant organisms that eventually resulted in recurrence of TB, and subsequent death with resistant organisms.

There is no doubt that combined drug therapy for TB has saved millions of lives in the last decades, and TB incidence has slightly decreased in recent years [2, 3]. Still, some 9 million cases of TB were detected in 2011 worldwide, especially in low and middle income countries [4]. Two factors have seriously jeopardized the TB problem. First, the emergence of multidrug resistant (MDR) TB in the early 1990ties in New York and San Francisco were the harbinger of a new epidemic of MDR-TB. In addition, TB patients co-infected with Human Immunodeficiency Virus (HIV) constitute a serious threat to global health, as HIV co-infection tremendously increases susceptibility to develop TB. Today, MDR-TB is alarming especially in Eastern European countries [5]. MDR-TB is defined by resistance to isoniazid and rifampicin, the two most effective anti-tuberculous first line drugs. Extensively drug-resistant tuberculosis (XDR) is defined as MDR-TB with additional resistance to a fluoroquinolone and injectable second-line therapy including capreomycin, kanamycin or amikacin [6]. Treatment of MDR-TB and XDR-TB is challenging; to date only treatments including rifampicin in combination with pyrazinamide have been shown to have sterilizing capacity when used in a short-course (i.e., six- months' duration) regimen. By necessity, MDR-TB and XDR-TB required prolonged treatment; current WHO guidelines now recommend 20 months of treatment, with at least 6 months' duration of injectables if *in vitro* susceptibility has been demonstrated. With increasing loss of susceptibility to the remaining agents, the chances of sputum conversion, and finally cure, diminish. Indeed, new effective anti-TB drugs are urgently needed. Recently, bedaquiline (TMC207) was

approved by food and drug administration (FDA) although, because of concerns about safety, a decision by European Medicines Agency (EMA) was postponed. Delamanid was recently approved by the EMA. Other new compounds currently being evaluated are PA-824, SQ109, PNU-100480 and AZD5847 [7].

Unfortunately, development of these new anti-TB drugs carries certain unknown risks of failure, even in late stages of development; and definitely it takes considerable time– around 12 years – before they finally reach the market and even after introduction, several products may still show unanticipated toxicity if wider used, and may need to be withdrawn from the market like trovafloxacin and grepafloxacin because of hepatotoxicity and cardio toxicity respectively. Besides, the search for new drugs is expensive and the low and middle income countries that constitute 95% of TB cases around the world have limited resources to support such research [8]. In order to speed up novel treatments for MDR-TB, new uses of approved (for other indications) existing drugs that are already available in the market could have an important role in MDR-TB treatment [9,10].

One of these drugs is moxifloxacin, a new generation fluoroquinolone that showed activity against *M. tuberculosis*. Although moxifloxacin is not registered for this indication, this drug is recommended for MDR-TB treatment [11,12]. Another new fluoroquinolone is gatifloxacin [13].

One of the drugs available in the market that is a potential candidate drug for TB is co-trimoxazole (SXT) because it is inexpensive, well tolerated and available worldwide but more importantly also in low and middle income countries [14]. SXT is a synergistic combination of trimethoprim (TMP) and sulfamethoxazole (SMX) in a ratio of 1:5. Both TMP and SMX are folic acid antagonist agents [15].

After oral administration, TMP is almost completely absorbed within 2-3 hours while the total absorption of SMX is 85-90%. Both of SMX and TMP are distributed in tissues that may be affected by TB like lung and brain. Following distribution, 60-80% of a total TMP appears in urine in an unchanged form and the rest are metabolites. Two of these metabolites are excreted in free and glucuronide forms, the latter, constitute 10-15% of total TMP concentration in the blood. For SMX, 60-65% of a total SMX is metabolized as the N^4 – acetyl derivative and 15% as N^1 glucuronide and these metabolites constitute 70% of total SMX in urine and 20-35% in plasma. Because of the relative long half-lives of SXT components, this drug is given every 8-12 hours for labeled indications [16].

SXT is prescribed for treatment of different diseases including urinary tract and respiratory infections. In patients with HIV, SXT provides prophylactic and therapeutic activity against *Pneumocystis jiroveci* pneumonia (PCP) [17-21]. The drug is available as tablets, oral suspension and parenteral formulation which is very convenient to treat patients in different stages of illness.

In general, a number of pharmacokinetic (PK) in relation to pharmacodynamic (PD) parameters predict the efficacy of antimicrobial agents [22]. They include the ratio of area under the concentration-time curve (AUC) from 0 to 24 relative to the minimum inhibitory concentration (MIC) (AUC/MIC) and the duration of time a drug concentration remains above the MIC (t>MIC) [23]. Because PK/PD studies are relative new, an old drug like SXT lacks data compared to newer drugs because these PK/PD studies have not been performed yet. Only *in vitro* data regarding SXT against *Bukholderia pseudomallei* are available and these data show that AUC/MIC ratio should exceed 25 [24]. As SXT is used for long-term prophylaxis of PCP HIV infected patients, the emergence of resistance among a range of pathogens has unfortunately been observed [25,26].

Limited data are available on the use of SXT for TB. Drug susceptibility data are scare, mechanism of resistance is unknown, little is known about the PK of SXT in TB patients and PK/PD parameters of SXT against *M. tuberculosis* are also unknown. Therefore, it is a challenge to establish a suitable dose of this drug for TB, especially in case of MDR-TB.

OBJECTIVES

The general aim of this thesis is to explore the potential use of SXT for TB treatment by evaluating pharmacokinetic parameters in TB patients, drug susceptibility against *M. tuberculosis* (PD) and PK/PD parameters of this drug.

OUTLINE

Chapter 1. In the first chapter we reviewed the *in vitro*, *in vivo* and clinical anti-tuberculosis (anti-TB) activity of six antimicrobial drugs (thioridazine, metronidazole, doxycycline, disulfiram, tigecycline and co-trimoxazole). These drugs are already available in the market and are not listed in WHO guidelines on MDR-TB treatment. These drugs may prove to be potential candidates for the treatment against *M. tuberculosis* or may serve as a lead for future drug development.

Chapter 2. Based on drug susceptibility tests and lacking other active antimicrobial drugs, SXT has been administered to individual patients in our center. Due to lack of the PK and PD parameters of this drug in MDR-TB patients, we retrospectively evaluated these parameters for SMX (the effective component of SXT against *M. tuberculosis*) along with its tolerability and safety. To further explore the PK in TB patients and compare it with other patients, a population PK model (POP-PK) would be developed to be useful for the assessment of potential altered PK parameters in MDR-TB patients.

Chapter 3. To assess the susceptibility of *M. tuberculosis* isolates to SMX among different TB patients, we selected isolates from two groups of TB patients: (normal sensitive TB and HIV-co infected TB). Consequently, we compared the susceptibility of these isolates to SMX with that of MDR-TB patients from the retrospective study (chapter 2).

Chapter 4. In order to measure the concentrations of SMX and its toxic metabolite in plasma or serum to find the relationship between the concentration of SMX and toxicity of its metabolites and efficacy of SMX, we developed a new analyzing method liquid chromatography mass spectrometry (LC-MS/MS).

Chapter 5. Using the developed LC-MS/MS method, we performed a prospective study to measure the PK and PD of SMX in normal sensitive TB patients. Furthermore, we calculated the values of f AUC/MIC ratio and T>MIC to be as a reference to measure the efficacy of SMX part of SXT and subsequently to find the suitable dose in the treatment of TB.

In **Chapter 6** we developed and validated dried blood spot (DBS) as a method to collect the blood samples. DBS is necessarily needed to quantify the concentrations of SMX and its metabolites for therapeutic drug monitoring.

The last section of the thesis, **Chapter 7**, includes summary, conclusion of important findings, general discussion, limitations of the research and their effect on the research results and future prospective.

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CHAPTER 1

POTENTIAL ANTIMICROBIAL AGENTS FOR THE TREATMENT OF MDR-TB

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Potential antimicrobial agents for the treatment of MDR-TB

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ABSTRACT

Treatment of multidrug-resistant tuberculosis (MDR-TB) is challenging because of the high toxicity of second-line drugs and the longer treatment duration than for drug-susceptible TB patients. In order to speed up novel treatment for MDR-TB, we suggest considering expanding the indications of already available drugs. Six drugs with antimicrobial activity (phenothiazine, metronidazole, doxycycline, disulfiram, tigecycline and co-trimoxazole) are not listed in WHO guidelines on MDR-TB treatment but could be potential candidates for evaluation against *M. tuberculosis*.

A systematic review was conducted to evaluate antituberculous (anti-TB) activity of these drugs against M. tuberculosis. We searched PubMed, Google scholar and Embase for English articles published up to December 31, 2012.

We reviewed in vitro, in vivo and clinical anti-TB activity of these drugs in addition to pharmacokinetics (PK) and side effects. Of the drugs effective against active replicating TB, co-trimoxazole seems the most promising one because of its consistent pharmacokinetic profile, easy penetration into tissue and safety profile. For the dormant state of TB, thioridazine may play a potential role as an adjuvant for treatment of MDR-TB. A strategy consisting of PK/PD studies, dose finding and phase III study is needed to explore these drugs for their role in MDR-TB treatment

INTRODUCTION

Multidrug-resistant (MDR) tuberculosis (TB) is defined as an infectious disease caused by *Mycobacterium tuberculosis (M. tuberculosis)* that is resistant to the two most powerful drugs currently known: isoniazid and rifampin [1-4]. Treatment of MDR-TB is complex, using toxic drugs that must be administered for a longer duration than for drug-susceptible TB patients, and with lower likelihood of treatment success [5].

The loss of rifampin in the treatment regimen of MDR-TB is particularly important because although many drugs have the potential to kill rapidly dividing metabolically active mycobacteria, few drugs are active when the population of *M. tuberculosis* has switched its genetic program to a quiescent, dormant phenotype. These persisters provide the major challenge for the immune system as well as for drug treatment to achieve sterility [6].

WHO estimates that 2.5% of all TB patients and 3% of all new cases are infected with MDR-TB [7,8]; an estimated 650,000 prevalent cases of MDR-TB occurred globally in 2010 [9]. The proportions of new TB cases with MDR-TB at the country level in Eastern European countries range at an alarming level of 19.4-32.3% [10]. One recent report from Belarus even indicates that almost half of newly diagnosed treatment-naïve patients with TB actually have MDR-TB. Further, the fact that patients < 35 years of age showed twofold higher odds of MDR-TB than those aged ≥35 suggests that emergence of MDR-TB is rampant in Belarus [11].

Most of the drug resistance is caused by mutations in the genome of *M. tuberculosis* coding for drug targets, but there is also evidence for mutations resulting in upregulation of bacterial efflux pumps, potentially reducing susceptibility for several drug groups [12, 13]. An important feature of *M. tuberculosis* is that under stress conditions, including hypoxia and host factors like nitric oxide, the organism is able to change its genetic program by e.g. inducing a 48-gene regulon via the response regulator DosR; this leads to inhibited aerobic respiration, thereby suppressing *M tuberculosis* replication [14].

It is clear that new antituberculous drugs are urgently needed. Unfortunately, development of new drugs takes time, is difficult and expensive, and low- and middle-income countries that comprise 95% of TB cases around the world have limited resources for health expenditure as well as lack of support for research [15]. In order to speed up novel treatments for MDR-TB, in this paper we explore the possibility of expanding the indications of drugs already available on the market.

We performed a systematic review of the published literature to select and describe antimicrobial drugs that are not listed in WHO guidelines on MDR-TB but may have *in vitro* and/or *in vivo* or clinical antituberculous activity, and are already available on the market labelled for other diseases. Based on these data, in addition to pharmacokinetic

parameters, administration route, safety and tolerance of the selected drugs, suggestions are made as to which drug is promising so that it can be further evaluated for MDR-TB treatment.

SEARCH STRATEGY AND SELECTION CRITERIA:

To select antimicrobial drugs for our systematic review that have activity against *M. tuberculosis,* we used the following search strategy in Pubmed: antimicrobial drugs AND tuberculosis AND 2012. To assess the activity of the selected drugs, we performed a second search in the databases PubMed, Google scholar and Embase for published, original and relevant literature with search terms that included but weren't restricted to the name of each drug combined with *M. tuberculosis*. Selection criteria were activity of the selected drug against *M. tuberculosis in vitro, in vivo* and/or clinical studies. *In vitro* studies that described susceptibility to the drug of interest were selected. *In vivo* also included animal studies infected with *M. tuberculosis* describing the log decline in colony-forming units CFU of *M. tuberculosis* per kilogram of body mass following drug administration. Clinical studies included clinical trials that described efficacy, sputum culture conversion and clinical outcome in MDR-TB patients. Further cross-references were obtained manually from bibliographies of identified and relevant papers and books. English-language publications covering all dates from the creation of each database up to December 31, 2012 were included. To evaluate relevant pharmacokinetic parameters, safety and tolerance of the drugs, studies in TB patients were selected. If the latter were not available, representative data of the drugs were presented. The results were divided per drug, using within each case the subheadings *in vitro, in vivo* and clinical data. A flow chart is presented in figure 1.

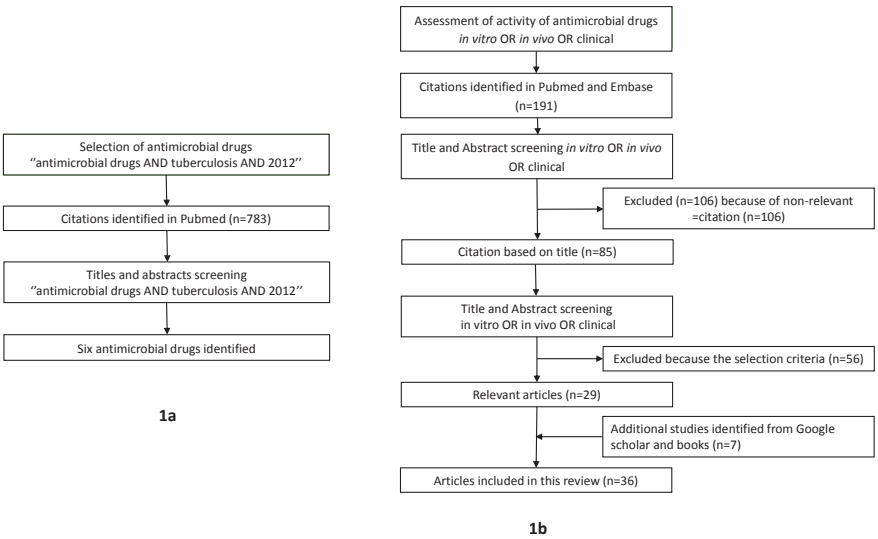

FIGURE 1- Flow chart of the selection process of retrieved publications: (a) selection of antimicrobial drugs, (b) selection of publications

RESULTS

Based on the first search strategy, 783 publications were retrieved. Screening of the titles and abstracts of these publications resulted in six antimicrobial drugs that are available on the market and not listed for tuberculosis in WHO guidelines. The selected antimicrobial drugs were phenothiazines (thioridazine and chlorpromazine), metronidazole, tetracyclines, disulfiram and co-trimoxazole (sulfamethoxazole-trimethoprim).

Our second search strategy resulted in 191 published articles. Based on screening of the titles 85 articles were selected, and 106 articles were excluded because of non-relevant titles. The abstracts of 85 articles were screened; 56 were excluded because the selection criteria weren't met. Finally, 29 relevant articles in addition to 4 articles form Google scholar and 3 references from the books were included in the systematic review. The search in Embase did not result in additional articles compared to PubMed. The results for each drug are discussed in detail below. A summary of the results, based on *in vitro*, *in vivo* and clinical data is provided in Table 1. Mechanisms of actions of these drugs are described in Figure 2. Pros and cons of the selected drugs are summarised in Table 2. The clinically evaluated dose and the effect of the drugs against TB are summarised in Table 3.

TABLE1-Summary of *in vitro*, *in vivo* and clinical activity of drugs against *M. tuberculosis*

Group	Drug	Route	Activity								Recommendation
			in vitro			*in vivo*		*clinical data*			
			activity	MIC	reference	activity	reference	activity	Pharmacokinetics	reference	
Phenothiazine	Thioridazine	oral	+	2.5 µg/ml	[21, 22]	+	[23]	+#	Wide distribution into tissues.	[24, 25]	Clinical trials are needed to investigate the suitable dose for treatment of cavitary pulmonary *M. tuberculosis*.
Nitroimidazole	Metronidazole	oral	+/–#	N.A.	[34,38, 39]	–	[38, 41-43]	+ #	Good penetration into CNS and CSF.	[37]	Further clinical studies are required. Treatment of TB meningitis.
Tetracyclines	Tigycycline	IV	–	8-64 µg/ml	[58]	N.A.		N.A.	High AUC in ELF and good penetration in the lung.	[52, 59]	*In vitro, in vivo* and clinical studies against slow-growing *M. tuberculosis*.
	Doxycycline	oral	+/–	2.5 µg/ml	[54, 57]	+	[54]	+	Good penetration in the brain and sufficient concentration in lung interstitium.	[52]	Treatment of TB meningitis.
Sulfhydryl (-SH, thiol)	Disulfiram	oral	+	8 µg/ml	[65, 67]	+	[64, *65*]	+	Wide distribution into lipids of tissues and highest level in skeletal muscle.	[70]	Further clinical studies, especially pharmacokinetics.
Sulfonamide	Co-trimoxazole	oral	+	1/19 µg/ml	[74, 76, 78-80]	N.A.		+	Distributed mostly in CSF.	[81]	Good candidate for treatment of TB meningitis. *In vivo* studies are required.

+: selected drug has bactericidal activity; –: selected drug has no bactericidal activity; +/–: selected drug has bactericidal activity in one study and no activity in other studies; #: anti-TB activity of selected drug in combination with anti-TB regimen; N.A: not available.

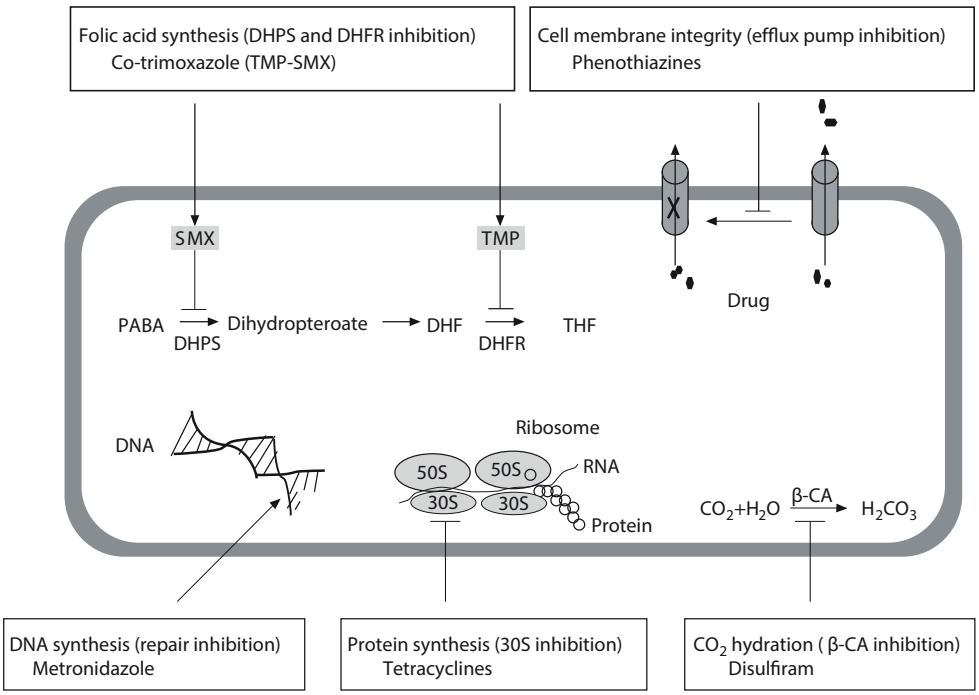

FIGURE 2-The mechanisms of action of the selected drugs. PABA: p-aminobenzoic acid; DHPS: dihydropteroate synthase; DHF: dihydrofolate; DHFR: DHF reductase; THF: tetrahydrofolic acid; SMX: sulfamethoxazole; TMP: trimethoprim; β-CA: β-class carbonic anhydrase

TABLE 2- Pros and cons of six selected antimicrobial drugs

Group	Drug	Pros	Cons	ref
Phenothi-azine	Thioridazine	Inhibition of growth of MDR-TB Treatment of intracellular MTB Adjuvant with other anti-TB drugs	Not useful for treatment of cavitary pulmonary MTB Increasing the risk of Torsade Pointes with subsequent risk of sudden death Less distribution to the brain Almost entirely bound to plasma protein	[19-21, 26, 28]
Nitroimida-zole	Metronida-zole	Good bactericidal action against dormant TB bacilli Good penetration into CNS and CSF [37] Beneficial adjuvant role in TB treatment Excellent oral bioavailability Well tolerated	Not effective against rapidly growing MTB. Not preferable for administering metronidazole to alcoholic patients	[33-35, 37, 44, 49, 51]
Tetracyclines	Doxycycline	Good penetration in the brain and sufficient concentration in lung interstitium [52] Safe in human Well absorbed	Bacteriostatic, not bactericidal against MTB	[52, 54]
	Tigecycline	Active against rapidly growing MTB High AUC in ELF and good penetration in the lung No serious side effects Longer half-life than doxycycline	Bacteriostatic antimicrobial drug Not active against slowly growing MTB High MIC (8-64) µg/ml Available only in injectable form No clinical and in *vivo* data	[52, *55*, 56, 58, 59, 62]
Sulfhydryl (-SH, thiol)	Disulfiram	Bactericidal activity in vitro and in vivo No cross resistant to other anti-TB drugs Active against persisters of MTB and enhanced the activity of rifampin and pyrazinamide Completely absorbed and widely distributed in the tissues	Poor clinical studies for TB indication	[64, 65, 70]
Sulfonamide	Co-trimoxazole	Good penetration into CNS and CSF Penetrate ELF easily Long half life Safe and well tolerated Consistent pharmacokinetic profile Cheap Highly susceptible MTB strains Synergies with other anti-TB drugs Preventing emergence of resistance of MTB when added to isoniazid or rifampin	*in vivo* study is not available	[77-81, 84, 85]

MTB: *M. tuberculosis*; ELF: epithelial lining fluid; AUC: area under the curve; CSF: cerebrospinal fluid; CNS: central nervous system

TABLE 3- Clinical studies showing response of TB patients to the selected drugs

Drug	Treatment regiment	Patient population	Dose regimen	Response	ref
Thioridazine	In combination with linezolid and/ or moxifloxacin	XDR-TB patients	25 mg ×2 q.wk → 25 q. wk→200mg q.d	earlier bacteriological sputum conversion relapse-free cure	[29]
Metronidazole	Anti-TB regimen (streptomycin, isoniazid and rifampin)	advanced pulmonary TB patients	400 mg×3q. d for 8 wk	significant improvement in clinical response reduction of sputum quantity enhanced radiographic improvement improvement in susceptibility to anti-TB drugs	[49]
Doxycycline	-----	TB patients	20mg×3q. d	suppress immunopathologic MMPs→ reducing tissue damage	[54]
Tigecycline	-----	-----	----	No clinical data available	-----
Disulfiram	-----	-----	----	-----	----
Co-trimoxazole	with Anti-TB regimen	MDR-TB patients	480 mg, 960 mg q. d	No treatment discontinuation No serious side effects	[77]

Anti-TB: Antituberculous; TB: tuberculosis; XDR-TB: extensive drug resistance tuberculosis; MDR-TB, multidrug resistance tuberculosis; Q: per; wk: week; d: day

PHENOTHIAZINES - THIORIZADINE

Phenothiazines such as thioridazine, chlorpromazine and promethazine belong to the group of anti-psychotic drugs [16]. The phenothiazine derivative thioridazine is a neuroleptic compound that has been used for over four decades [17]. Phenothiazines have the ability to inhibit the bacterial efflux pump which protects the bacterial cell against harmful substances to which the cell is exposed [16]. Thioridazine not only inhibits specific efflux pump systems but also the expression of genes that code for efflux pumps of *M. tuberculosis* [18].

In vitro studies

Phenothiazine family compounds, including chlorpromazine and thioridazine, are known to possess appreciable levels of antimicrobial activity against MTB organisms *in vitro*. The anti-MDR-TB activity was similar in thioridazine and chlorpromazine [19]. These compounds may be concentrated more than tenfold by macrophages that phagocytise TB tuberculosis bacilli [20]. It is likely that the concentration of either phenothiazine required to kill *M. tuberculosis* cells *in vitro* is achieved not only because the macrophage is able to concentrate phenothiazines but also to make these compounds available in an active form to the cytoplasmic structure of the macrophage that houses the entrapped phagocytised bacterium [19]. Thioridazine inhibits the growth of clinical isolates of *M. tuberculosis* that are resistant to streptomycin, rifampin, isoniazid, ethambutol and pyrazinamide (first-line anti-TB drugs) [21]. Minimum inhibitory concentration of thioridazine required for 50% inhibition of *M. tuberculosis* H37Rv clinical isolates is 2.5 mg/L [22]. Because thioridazine does not appear toxic to the macrophage *in vitro*, it could be used in the treatment of intracellular *M. tuberculosis* infections [19].

In vivo studies

Groups of five female BALB/C mice were infected intraperitoneally with 10^6 colony-forming units (CFU) /mL of *M. tuberculosis* and treated with thioridazine in a dose range 0.05-0.5 mg/day, which is equivalent to that used for psychosis in humans (1200 mg/day). There was a higher-than-5 log reduction in the number of CFU/kg derived from the lungs of infected mice compared to the control group within one month [23].

Clinical studies

When thioridazine is administered orally it is rapidly absorbed, with peak plasma concentrations occurring within 2-3 hours. Thioridazine is widely distributed in tissues like liver, blood and kidney [24, 25], yet it is distributed less favourably to the brain [26]. It is not useful for treatment of cavitary pulmonary *M. tuberculosis* because the concentration of thioridazine required for killing or inhibiting *M. tuberculosis* outside the macrophage exceeded the concentration that could be achieved in patients receiving standard dosages [19].

Thioridazine undergoes 5-sulfoxidation and *N*-demethylation by CYP1A2 and CYP3A4, while CYP2D6 catalyses mono-2- and di-2-sulfoxidation of thioridazine in the human liver. CYP2D6 and CYP3A4 catalyse thioridazine mono-2-sulfoxidation [27]. Thioridazine is almost entirely (99.85%) bound to serum proteins; its metabolites are bound to serum proteins to a lesser extent. This could be a problem because a small change in the binding capacity of proteins can alter the unbound concentration of thioridazine that has clinical activity. The unbound concentrations of thioridazine metabolites that include thioridazine side-chain sulfoxide and thioridazine side-chain sulfone are higher (2 and 9 times respectively) than the unbound concentration of thioridazine [28].

Thioridazine can be used as adjuvant for regimens already containing several other drugs for treatment of MDRTB. It is administered in drug-resistant TB until the susceptibility of strains is known [20].

Of 17 XDR-TB patients, 14 were treated with thioridazine in combination with linezolid and/or moxifloxacin at a daily dose of only 25 mg for 2 weeks, after which the dose was increased by 25 mg weekly until it reached 200 mg/day. The combined therapy of thioridazine, moxifloxacin and linezolid cured 61% of patients, and 22% of patients who were still on follow-up showed beneficial response. Thioridazine was discontinued in two patients because of pancytopenia in one patient and allergic dermatitis in the other. In this study, the authors speculate that thioridazine could have contributed to an earlier bacteriological sputum conversion. No prolongation of the QT interval or other cardiac complication was observed in those patients who received thioridazine. Combined therapy including linezolid, moxifloxacin and thioridazine was associated with a relapse-free cure in most cases [29]. The quality of life of the patients was improved shortly after the inclusion of thioridazine in their regimens; most of them benefited from less night sweating, increased appetite, weight gain and decreased levels of anxiety [29]. In another study, thioridazine cured 10 out of 12 patients; the other two patients responded too but they dropped out of the program [30].

The side effects of thioridazine, as for all phenothiazines, are dose-dependent and include

QTc prolongation, thereby increasing the risk of Torsade Pointes with subsequent risk of sudden death [31]. To avoid the risk of sudden death in patients whose QTc is significantly increased, patients should be screened by ECG before and during treatment with thioridazine [30]. Because chlorpromazine causes frequent and serious side effects when administered chronically, it is not a good candidate drug for treatment of MDR-TB [32].

METRONIDAZOLE

Metronidazole is currently licensed for the oral treatment of infections like protozoa (trichomoniasis, amaebiasis) and anaerobic bacteria [33]. This drug has only been evaluated to a limited extent for TB [33], showing good bactericidal action in anaerobic conditions against dormant TB bacilli, but has no effect on aerobic cultures of *M. tuberculosis* [33- 35].

Bactericidal activity of metronidazole depends on the formation of a redox intermediate metabolite from the reduction of the nitro group in metronidazole under anaerobic conditions. This metabolite oxidises DNA and causes extensive breakage of DNA strands and subsequent cell death, and also inhibits DNA-ase1, which has a function as a repair endonuclease in bacteria. Hence reduced metronidazole exerts a dual action by breaking DNA strands and inhibiting the enzyme responsible for repairing strand breaks in DNA [34, 36, 37].

In vitro studies

Metronidazole showed no effect when added to bone marrow-derived macrophages infected with *M. tuberculosis* and did not decrease the bacterial load, although a high concentration of metronidazole was used [38]. Adding metronidazole to a regimen of rifampin, moxifloxacin and amikacin and/or capreomycin significantly improved killing of dormant (anaerobic and drug-tolerant) *M. tuberculosis* in an adipocyte model [39].

An *in vitro* study was conducted to determine the effect of metronidazole against tubercle bacilli incubated under aerobic conditions. Although a high concentration of metronidazole was used (512 µg/ml), it had no effect on aerobic cultures of *M. tuberculosis*. However, under anaerobic conditions, when 32 µg/ml of metronidazole was incubated with *M. tuberculosis* alone and in combination with either rifampicin or isoniazid, this caused a reduction of 69% and 95% in CFU/ml respectively. Again in this study, 8 days of anaerobic exposure of dormant bacilli to 8 µg/ml of metronidazole caused 2.7 log reductions in the number of CFU. Rifampicin enhanced the bactericidal activity of metronidazole when used in combination, leading to a 3.68 log unit decline in CFU/ml [34].

In vivo studies

Although metronidazole had no effect on the growth of *M. tuberculosis* in lungs of aerosol-infected mice at a dose of 15 mg/kg, there was a relatively small but statistically significant reduction in bacterial counts during a chronic phase of disease. This chronic phase, in which no remarked changes in bacterial load can usually be detected, and established after the onset of acquired cellular immunity of mice as a result of the progressively growing *M. tuberculosis*, resulted in a proportion of bacilli shifting into a dormant state [38, 40]. The possible explanation for the low activity of metronidazole is that *M. tuberculosis* is not in a state of anaerobic metabolism in which it is susceptible to metronidazole [38].

In another *in vivo* study using a granuloma model of *M. tuberculosis* dormancy (mouse hollow-fibre model), metronidazole (100 mg/kg) failed to show a beneficial effect against bacilli, although there was immunohistochemical and mutant survival-based evidence of tissue hypoxia. The possible explanations for this result could be a less-than-optimal concentration of metronidazole, poor penetration into granulomatous lesions or lack of hypoxic conditions to permit reductive activation of metronidazole [41].

Activity of metronidazole at 50 and 100 mg/kg in guinea-pigs infected with *M. tuberculosis* combined with standard regimens showed no significant activity, perhaps because of poor penetration of metronidazole into the necrotic core of the hypoxic granulomas or the concentrations of metronidazole effective against *M. tuberculosis* under completely anaerobic and microaerophilic conditions [42]. These results were comparable to the *in vivo* study, where treatment of BALB/c mice and C3HeB/FeJ mice that developed highly organized necrotic lesions following a TB infection called Kramnik model with 200 mg/kg metronidazole for 7 to 8 weeks had no bactericidal activity against *M. tuberculosis*, although necrotic lesions in the Kramnik model showed evidence of hypoxia [43].

Clinical studies

The oral bioavailability of metronidazole is almost complete (98.9%) [44] . Peak serum levels are reached within 1-3 hours [45]. Metronidazole is bound 10-20% to plasma proteins [45]. The half-life of metronidazole is about 8h [44]. Metronidazole is distributed in different tissues with various percentages of penetration, and it has good penetration into the cerebrospinal fluid (CSF) and central nervous system (CNS) [37]. It is metabolised in the liver, resulting in the formation of two oxidation products: the alcohol metabolite 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole, which has an antimicrobial activity of 30-75% compared to metronidazole, and the acidic metabolite 2-methyl-5-nitroimidazole-1-acetic acid, which has 5% activity of the metronidazole and is only detected in patients

with renal dysfunction [46, 47]. Metronidazole and its metabolites are mainly excreted in urine [48].

There are few clinical studies of metronidazole for TB. In a single-blinded study, metronidazole (400 mg three times daily) or a placebo were administered for the first eight weeks in addition to the standard anti-TB regimen. Addition of metronidazole resulted in a significant improvement in clinical response, with reduction of sputum quantity, enhanced radiographic improvement and improved sensitivity to anti-TB drugs compared to the placebo group. The results showed a 56% improvement in the sensitivity of tubercle bacilli to anti-TB drugs on follow-up over 12 weeks in the metronidazole group, compared to 38% in placebo group. This study confirmed that metronidazole has a beneficial adjuvant role in the treatment of TB [49].

In an ongoing clinical trial, metronidazole is used in MDR-TB patients at a dose of 500 mg three times daily in combination with standard second-line antituberculous drugs. The results of that study are to be expected soon [NCT00425113].

Metronidazole is generally well tolerated. Adverse reactions include reversible neutropenia, minor gastrointestinal side effects, metallic taste, vaginal and urethral burning, and darkening of the urine. Central nervous system side effects include ataxia, vertigo, peripheral neuropathy and headache [50]. Metronidazole can produce a reaction similar to that of disulfiram when administered to patients using alcohol because the interaction between metronidazole and ethanol leads to a toxic accumulation of acetaldehyde in the blood [51]. Care should be taken in using this drug for alcohol abuse patients.

TETRACYCLINES

Doxycycline and tigecycline belong to the tetracycline group of antimicrobials, which exhibit a broad spectrum of activity against different pathogens, including Gram-positive and Gram-negative bacteria [52]. Tetracyclines are bacteriostatic and act by binding to the bacterial 30S ribosomal subunit and inhibiting protein synthesis. Tetracyclines are effective in the treatment of a wide range of infectious diseases like community-acquired respiratory tract infection, sexually transmitted diseases and skin conditions [52].

Doxycycline is an antibiotic with broad spectrum matrix metalloproteinase (MMP) inhibitory activity [53]. It not only reduces the expression of MMPs, thereby minimising tissue damage in TB, but also suppresses mycobacterial growth. Because doxycycline is safe, cheap and almost universally available, it may represent a new adjunctive therapy to improve outcomes and reduce mortality for TB [54]. Tigecycline (GAR-936) is a new semisynthetic glycylcycline (tetracyclines analogue) and is effective against

intra-abdominal, skin and soft-tissue infections caused by staphylococci, enterococci or streptococci, as well as most enterobacteriaceae and anaerobic pathogens [52, 55]. Tigecycline is effective against rapid-growing mycobacteria (*M. fortuitum, M. chelonae and M. abscessus)* but showed no activity against more slow-growing mycobacteria (*M. tuberculosis)* [55, 56].

In vitro studies

Doxycycline suppressed MMP1 and MMP3 as well as TNFα secretion from primary human macrophages infected with *M. tuberculosis* at 72 h in a dose-dependent manner. This drug is bacteriostatic to *M. tuberculosis* clinical isolates with an MIC of 2.5 μg/ml [54]. In 69 MDR-TB isolates obtained from patients in the Samara region of Russia, 5 (7.4%) isolates were resistant to doxycycline [57]. In *vitro* MIC values of tigecycline against clinical isolates of *M. tuberculosis* were as high as 8-64 μg/ml [58].

In vivo studies

Doxycycline decreased mycobacterial replication in infected guinea pigs but showed no effect on MMP activity. In a dose of 5 and 20 mg/kg, doxycycline monotherapy suppressed lung CFU at 10 weeks in a dose-dependent manner. The results showed that doxycycline improved the outcome for TB by acting directly on mycobacterial proliferation rather than on MMP activity [54]. No publications mentioned the antimicrobial activity of tigecycline *in vivo.*

Clinical studies

Doxycycline is available in oral form, unlike tigecycline, which is only available as an injectable [52]. This drug is absorbed well from the gastrointestinal tract, with bioavailability ranging between 75-100% [52]. It is absorbed quickly and reaches its maximum serum concentration (Cmax) within 4 h [52]. Cmax and area under the plasma concentration time curve (AUC) of tigecycline are proportional with the dose [52, 55]. In epithelial lining fluid the area under the curve of tigecycline was 2.28 μg. h/ml, which is higher than that of serum, 1.73±0.64 μg. h/ml [59]. Doxycycline is lipophilic and penetrates well into tissues, especially the brain, eye, prostate and intestinal epithelia [52]. Higher concentrations of doxycycline are found also in the kidney and liver [60]. Tigecycline has a large volume of distribution, 710L/kg [61]. It shows good penetration into tissues like bones, skin, liver and lung [52]. The protein binding of doxycycline is between 60-95%, while that of tigecycline ranges between 71-89% [52].

Tigecycline has a half-life of 15-36 h, which is greater than the 12 h half-life of doxycycline [52, 59]. No more than 15% of tigecycline is excreted in the urine in unchanged form [52, 62]. About 30-40% of doxycycline is excreted unchanged in the urine [52].

Doxycycline is safe in humans and may suppress immunopathologic MMPs, thereby reducing tissue damage in TB patients using a low dose (20 mg twice daily). It may achieve sufficient concentration in the lung interstitium to decrease the growth of *M. tuberculosis* and modulate MMP activity and expression [54]. No clinical data are available for the potential use of tigecycline for the treatment of MDR-TB in humans. The most common side effects of doxycycline are gastrointestinal problems and skin reactions [63]. In a study with healthy subjects, tigecycline had no serious side effects except for nausea and vomiting, which were dose-related [62].

DISULFIRAM

Disulfiram (tetraethylthiuram disulfide, DSF) has been used orally in the clinical treatment of alcoholism since 1949. DSF is a prodrug and is enzymatically metabolised in the blood to metabolites, primarily diethyldithiocarbamate (DDC or DETC) within 4 min [64, 65]. DSF and DDC exhibit growth-inhibitory activity against bacteria, fungi, protozoa and viruses. DSF is effective against MDR/XDR-TB and exhibits bactericidal activity *in vivo* and *in vitro* [64]. The mechanism of action of DDC against *M. tuberculosis* has been reported as inhibition of beta-class carbonic anhydrases (beta-CAs) from *M. tuberculosis* [66].

In vitro studies

Peripheral blood mononuclear cells (PBMC) of healthy and HIV subjects were preincubated with 100-1000 ng /ml of DDC and then infected with *M. tuberculosis* H37Rv. DDC reduced the CFU number of these intracellularly-growing mycobacteria. DDC also enhances the antimycobacterial activity of monocyte-derived macrophages from healthy volunteers injected with 5mg/kg body weight DDC *ex vivo*. DDC can enhance macrophage maturation by the induction of 1, 25-$(OH)_2$ cholecalciferol (vitamin D3) [67]. Vitamin D3 may play an important role in the pathological process of tuberculosis by downregulating the levels of matrix metalloproteinases (MMPs) and upregulating the levels of tissue inhibitor of metalloproteinase (TIMPs) [68]. Vitamin D3 also mediates the production of cathelicidin, which is a monocyte-macrophage peptide with significant anti-TB activity, in the context of Toll-like receptor 2/1-ligand activation [69]. An *in vitro* study showed that DETC was highly active against tubercle bacilli with MIC of 8 µg /ml [65].

DSF and DDC showed antitubercular activity against more than 40 clinical isolates of *M.*

tuberculosis, including MDR/XDR-TB strains. The MIC90s of DSF and DDC against clinical isolates were 1.56 and 3.13 µg/ml respectively. They also show bactericidal activity against intracellular *M. tuberculosis* in human monocyte leukemia cell line (THP-1) at 6-30 µg/ml and 10-30 µg/ml respectively [64]. Since no cross resistance of DSF and DDC with other anti-TB drugs was observed, these compounds may be of potential value for future regimens against MDR/XDR-TB [64].

In vivo studies

DSF kills *M. tuberculosis* at (80-160) µg/kg in a mouse model with chronic TB [64]. DETC enhances the activity of pyrazinamide and rifampin twofold when co-administered in mice at 100 mg/kg, showing activity against persisters of *M. tuberculosis* [65].

Clinical studies

DSF is rapidly and completely absorbed following oral administration, and is quickly reduced to DDC. DDC is metabolised to diethylamine, carbon disulfide (CS_2), DDC methyl ester, DDC glucuronide and DDC sulfate; a small amount of DDC is reoxidised to DSF. DSF, DDC and CS_2 are widely distributed throughout the body in lipids of various tissues, and the highest levels of these compounds are found in skeletal muscle [70]. Humans eliminate more than 90% of orally administered DSF within 3 days by renal clearance as DDC and DDC glucuronide, to a small extent as DDC sulfate, and via the breath as CS_2 [70]. Few publications showed the activity of DSF or its metabolites against TB. Only one study found that DDC reduces the incidence of infections in HIV co-infected TB patients, probably by stimulating the antimicrobial activity of mononuclear phagocytes [67].

The side effects of disulfiram are mainly on the central nervous system and include psychosis or confusional state that occurs in the early period of DSF therapy with higher dosages of DSF (500 mg/day). Another serious side effect is peripheral neuropathy. All these side effects were reversible [71]. Rarely, DSF can cause fatal hepatitis [71].

CO-TRIMOXAZOLE

Co-trimoxazole (SXT) is a synergistic combination of two antimicrobial agents: trimethoprim (TMP) and sulfamethoxazole (SMX). Its mechanism of action is interference with folic acid synthesis of bacteria. SXT is used predominantly to treat urinary tract infections and for prophylaxis and treatment of *Pneumocystis jiroveci* pneumonia (PCP) in HIV patients [72, 73]. There is ongoing debate about the use of SXT for the treatment of TB. Some studies

mention that only SMX was effective against *M. tuberculosis*, while TMP is not [74, 75].

In vitro studies

M. tuberculosis strains appeared to be susceptible to SXT in 43 of 44 (98%) isolates tested. These isolates were sensitive to TMP-SMX at MIC ≤1/19 µg/ml [74]. Another study showed that SMX inhibits 80% and 99% growth of all 117 clinical isolates at MIC 19 mg/l and 38 mg/l respectively [76]. Drug susceptibility testing in a recent study mentioned that MICs values of SMX for *M. tuberculosis* ranged between 4.75-25 µg/ml [77]. In 7H9 broth, *M. tuberculosis* was susceptible to SMX; MIC90 was 8 µg/ ml. In that study SMX achieved an excellent activity against *M. tuberculosis* [78]. One *in vitro* study showed that *M. tuberculosis* strain H37Rv was susceptible to SMX and not to TMP at MIC of 8.5 µg/ml. When SXT was added to an isoniazid- or rifampin-treated *M. tuberculosis* culture isolate, SMX with and without TMP was efficient at killing and preventing its growth, thereby preventing the emergence of drug resistance [79].

In an *in vitro* study, *M. tuberculosis* strains were exposed to either a TMP/SMX combination, SMX and TMP alone, or SMX in combination with first-line TB drugs (isoniazid, rifampicin and ethambutol). TMP had a negligible effect on the growth of *M. tuberculosis*, while SMX inhibited 80% of the growth at *4.75* mg/L. There was no synergistic activity between the TMP and SMX combination, but an additive effect was observed. SMX had a synergistic effect combined with rifampicin, an additive effect combined with ethambutol, and no effect with isoniazid [80].

Clinical studies

The total absorption of SMX is 85-90%. Concentrations of the non-protein-bound fraction of SMX to TMP in plasma varied between subjects, ranging from 1:5 to 1:40 [81]. The explanation could be that SMX has a smaller volume of distribution (10-20L) than TMP (69-133L). In the blood, SMX is bound to plasma proteins to an extent of 58-66% (34-42% free) [81, 82]. SMX is well distributed in most body fluids and also in cerebrospinal fluid, so it may be a good candidate for treating TB meningitis [81]. Concentrations of SMX in sputum, middle-ear fluid and paranasal sinus fluid are about 20-27% of serum, and in bronchial secretion 60-100% of those in serum [83]. SXT penetrates epithelial lining fluid easily because it is lipophilic and inflammation-independent [84, 85]. Following distribution of SMX, it is partially acetylated and glucuronide-conjugated in the liver [86]. SMX excretions are 60-65% acetyl derivative, and 15% appear as glucuronides. These metabolites constitute 70% of total SMX in urine and 20-35% in plasma [81].

The plasma half-life of SMX is 9 hours [81].

Limited data are available on the pharmacokinetic parameters of SXT in MDR-TB patients. Only one study described the PK parameters after receiving 480 mg and 960 mg of co-trimoxazole. PK parameters of SMX in TB patients including area under the curve, clearance and volume of distribution are lower than the values observed in patients with other indications. These patients seem to display a consistent PK profile for SMX [77].

Adherence and tolerance to the drug was good when SXT was used in a daily dose of 960 mg as prophylaxis to reduce the mortality in adults with HIV infection and TB, by preventing opportunistic infections. The rates of occurrence of one or several side effects that could be due to SXT were similar in the placebo group (n=372) and in the co-trimoxazole group (n=371) [87]. An SXT dose of 960 mg/day added to routine care improved the survival of HIV-positive TB patients dramatically; SXT prophylaxis should therefore be added to the routine care of HIV-positive TB patients [88].

In five clinical studies summarised in one study [73], co-trimoxazole was safe, feasible and effective in the prophylactic treatment of HIV patients with TB; it has beneficial effects like lower mortality, fewer hospital visits, weight gain, improved CD4 cell counts and reduced plasma HIV in PCP, malaria and other bacterial (pneumococcal) airway infections. The impact on infections with M. tuberculosis was not considered in these studies.

In general, TMP-SMX is a safe medication and is tolerated well. The most common side effects include gastrointestinal intolerance, nausea, vomiting, anorexia and diarrhea [86]. Possible side effects in the blood are hyperkalemia, slight increase in serum creatinine levels (not representing loss of glomerular filtration but rather reversible decrease in tubular excretion of the creatinine molecule) and hypernatremia. These effects occur especially in patients with renal dysfunction [86, 89, 90]. Hematological abnormalities include leucopenia, agranulocytosis and thrombocytopenia, hemolytic and aplastic anemia [91].

DISCUSSION

The need for new drugs to improve the treatment of patients with MDR-TB has received much attention. These patients are currently treated with a combination of second-line drugs that are more expensive, more toxic and less effective than the drugs used in standard therapy, and which have a much longer treatment duration than those for drug-susceptible TB patients. This often results in poor outcomes. Exploring the antimicrobial activity of drugs that are already available on the market would therefore be a tremendous asset. This review identifies the pharmacokinetics and pharmacodynamics and the *in vitro, in vivo* and clinical data of these drugs.

Based on the *in vitro, in vivo* and clinical data of the drugs discussed in this review, thioridazine, doxycycline, metronidazole and co-trimoxazole were selected as candidate drugs for possible use in MDR-TB. They are effective either against metabolically active, fast-replicating, or metabolically inactive, non-replicating phenotypes of *M. tuberculosis* (dormant state*)*. These drugs also have favourable pharmacokinetics, like CNS and CSF penetration, that may be useful for the treatment of TB meningitis.

However, thioridazine has no activity against rapidly-replicating metabolically active *M. tuberculosis*, such as inactive cavitary pulmonary TB, because of the poor penetration of this drug into the cavities; it thus lacks efficacy against this stage of TB. The same is true for metronidazole, which is predominantly active under anaerobic conditions, although this drug appears to have activity against dormant *M. tuberculosis*. Compounds of similar structure to metronidazole have been shown to have potent antitubercular activity *in vitro* and *in vivo*, like CG17341 and PA 824 [92]. Metronidazole could thus be used as a lead compound for the synthesis of new drugs against MDR-TB, especially for dormant organisms. Doxycycline has bacteriostatic but no bactericidal effect against *M. tuberculosis*, so it isn't the first choice to develop further.

According to the pros and cons of the selected drugs mentioned in detail in the Results section, co-trimoxazole could be the promising drug for treating MDR-TB because of its consistent pharmacokinetic profile in combination with other anti-TB drugs. It is an easy-to-administer, cheap, well-tolerated and safe antimicrobial agent. It has also encouraging activity *in vitro*. The other compounds discussed here have some limitations that may restrict their uses for treatment of MDR-TB. Because of the fatal outcome of a disulfiram-alcohol reaction, this drug should not be administered to TB patients who are alcohol abusers. Tigecycline is administered only parenterally and is not effective against more slow- growing mycobacteria such as *M. tuberculosis*.

Our review has several strengths. We conducted a very comprehensive literature search by exploring electronic databases to get information about anti-TB activity of the selected

drugs. These drugs are not listed in WHO guidelines but they have in *vivo, in vitro* or clinical activity against *M. tuberculosis*, and no previous review mentions their antimicrobial activity in the treatment of MDR-TB. Hence this review could be an interesting starting point for further research in the future.

This review has also some limitations. The review was restricted to only six antimicrobial drugs and does not discuss other new or already-available drugs that have anti-TB activity. Most published articles provide insufficient data for the role of these drugs in the treatment of tuberculosis. We excluded those articles not available in the English language, even though articles in other languages could have important data. Because limited MDR-TB clinical data for these drugs are available, a lack of dose selection studies was observed. We did however mention the doses and response to these drugs for TB and XDR-TB patients in clinical studies if they weren't available for MDR-TB patients.

There is only limited antimicrobial pharmacokinetic-pharmacodynamic (PK/PD) information available for the drugs discussed here. This could be an obstacle for finding suitable doses and predicting their efficacy in the treatment of MDR-TB patients. Antimicrobial PK/PD derived in preclinical PK/PD models like the hollow-fibre infection model and the mouse models, as well as additional clinical trials, do offer the possibility to study toxicity and to determine duration of therapy. These will be important steps in the further development of these drugs for use in MDR-TB.

A clinical prospective study should be conducted to evaluate pharmacokinetic parameters and tolerance in order to find the suitable dose for the treatment of MDR-TB patients in a phase II study. In a phase III study, the clinical efficacy and long-term safety of the drug should be investigated by comparing the sterilising activities of the candidate alone and when substituted for one standard anti-TB drug. These approaches are essential for an efficient clinical development of potential antimicrobial drugs for TB treatment.

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CHAPTER 2

EVALUATION OF CO-TRIMOXAZOLE IN TREATMENT OF MULTIDRUG-RESISTANT TUBERCULOSIS

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ABSTRACT

Co-trimoxazole (SXT), a combination of sulfamethoxazole (SMX) and trimethoprim has shown in vitro activity against *Mycobacterium tuberculosis*. However, the pharmacokinetic (PK) and pharmacodynamic (PD) parameters of SXT in multidrug-resistant (MDR) tuberculosis (TB) are so far lacking. Therefore, we evaluated the PK and drug susceptibility along with its tolerability during treatment.

Based on drug-susceptibility testing MDR-TB patients received SXT as a part of their MDR treatment. The PK parameters of SMX, the effective component of SXT against Mycobacterium tuberculosis were evaluated. The ratio of AUC0-24h/MIC was used as the best PK/PD parameter to predict the efficacy of SMX. Adverse effects of SXT were also evaluated.

Ten patients with MDR-TB (one of whom had XDR-TB) received 480 mg of SXT with median dose of 6.5 mg/kg of SXT (Range, 6.1-6.8) qd for a median treatment period of 381 days (Range, 129-465). In two patients, the dose was escalated to 960 mg. *f*AUC0-24/MIC of SMX exceeded 25 in only one patient. SXT was safe and well tolerated except for one patient who had gastrointestinal side effects after receiving 960 mg of SXT. Additional studies are needed to find the PK/PD targets and consequently to set the optimal dose of SXT for MDR-TB treatment.

INTRODUCTION

Multidrug-resistant (MDR) and extensively drug resistant (XDR) tuberculosis (TB) are emerging in many areas around the world [1]. Also the susceptibility of *Mycobacterium tuberculosis* against current antituberculosis (anti-TB) drugs has decreased and therefore the treatment of MDR-TB has become increasingly complicated [2]. Consequently, there is an urgent need for new effective drugs with a minimum of toxicity. An old, inexpensive and well-tolerated drug like co-trimoxazole (SXT), which is registered for other indications than TB, could be a new effective agent for the treatment of MDR and XDR-TB [3].

SXT is a combination of trimethoprim (TMP) and sulfamethoxazole (SMX) in a ratio of 1:5. It is a potent antibacterial drug against a variety of pathogens causing infections in humans. SXT is currently used in the treatment of urinary tract infections, otitis media, shigellosis, chronic bronchitis and *pneumocystis carinii* pneumonitis [4].

SXT shows concentration-independent or time dependent killing. Therefore, the ratio of area under the free concentration-time curve (***f***AUC) from 0 to 24 h relative to the minimal inhibitory concentration (MIC) is considered to be the important pharmacokinetic (PK)/ pharmcodynamic (PD) parameter to predict the efficacy of SXT [5]. Very little is known about the PD of SXT [5]. There are only few publications on the PK parameters of SXT of which none in TB patients (table1) [6-9]. Only two studies investigated the *in vitro* susceptibility of SXT against *M. tuberculosis* and showed promising results [3, 10]. From these studies it could be concluded that only SMX was effective against *M. tuberculosis a*nd TMP is not [3, 10,11]. One study mentioned AUC/MIC ratio of SXT that had to be exceeded 25 for effective treatment of melioidosis caused by *Burkholderia pseudomallei* [12] .The lack of data is likely due to the fact that SXT is an old drug and *in vitro* evaluation of PK/PD parameters in infection models is rather new.

In general, SXT is a safe and well-tolerated drug. Gastrointestinal complications including nausea, vomiting, anorexia and diarrhea are the most common adverse effects of SMX [13, 14]. Renal side effects including hyperkalemia, slight increase in the serum creatinine level and hyponatremia occur especially in patients with renal dysfunction [15, 14,16]. Other side effects reported are hematological side effects like megalo-blastic anemia, leucopenia, thrombocytopenia and aplastic anemia in patients with preexisting megaloblastic anemia or deficiencies in folic acid stores (alcoholics, malnourished patients and pregnant women) [17].

Although SXT has been administered to TB patients, data is very scarce and its role in TB treatment is still not yet clear. The objective of this study was to evaluate PK, PD and PK/ PD parameters and safety/tolerability of SXT in MDR-TB patients

TABLE1- Pharmacokinetic parameters of SMX for the treatment of different infections from previous studies

Infection	Subjects	Dosage regimen	T ½ (hr)	Vd (L/kg)	Cl (ml/ min/kg)	AUC (mg/L/hr)	ref
Bacterial skin disease	12	Single dose 1.6 g orally	10.0±1.1	N/A	N/A	1295±823	[9]
Normal meninges	9	25 mg/kg over 120 mini.v.	9.8±1.5	0.30±0.04	0.36±0.03	1,160±103	[6]
AIDS	8	75 mg/kg daily (IV)	15.5±7.4	0.5± 0.3	0.40±0.12	N/A	[7]
HIV	10	800 mg once daily orally	N/A	N/A	N/A	574.2 (342.6-796.3)	[8]

Data are presented as n, mean ±SD or median (range), t1/2: half- life; VD: volume of distribution; AUC: area under the concentration time curve; NA: not available

PATIENTS AND METHODS

Patients

MDR-TB patients who were referred to the Tuberculosis Center Beatrixoord of the University Medical Center Groningen (Groningen, The Netherlands) between the 1st January 2006 and the 1st July 2012 and for whom drug susceptibility testing (DST) for SXT was performed, were eligible for evaluation. Age, gender, weight, ethnicity, underlying disease, minimum inhibitory concentration (MIC) of the *Mycobacterium tuberculosis isolate*, localization of TB, other anti-TB medications, duration of treatment with SXT and the total anti-TB regimen administration were recorded for MDR-TB patients that received SXT. Patients were subjected to routine medical care without specific study-related interventions. We describe patient data obtained during usual care, and therefore, no ethical clearance was required under Dutch Law (WMO).

Pharmacokinetics and Pharmacodynamics

Blood samples were only evaluated when obtained at steady state which was after at least three days of administration of SXT [18]. They were collected before and at 1, 2, 3, 4 and 8 h after SXT administration.

The concentrations of SMX in human plasma samples were analyzed in the laboratory of Clinical Toxicology and Drugs Analysis of the Department of Hospital and Clinical Pharmacy at the University Medical Center Groningen by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS). In brief, 5 µl of each plasma sample was mixed

with 750 µL precipitation reagent (methanol and acetonitrile 4:21, v/v). From the clear upper layer 5 µL was injected on a 50 mm×2.1 mm reversed phase C18, 5-µm analytical column (HyPurity Aquastar, Interscience Breda, The Netherlands) for chromatographic separation. The detector was operated in electrospray positive ionization mode and performed selected reaction monitoring (SRM) as scanning mode. The transition of *m/z* 254.1-155.9 (collision energy 25 eV) for SMX was measured with scan width of 0.5*m/z*. The recoveries ranged from 97.7% to 102.9%, depending on the concentration. The accuracy was between 97.7% and 102.9% for SMX, depending on the concentration level. The intra- and inter assay coefficients of variation were less than 5.1 % over the ranges of 5 to 100 mg/L. The lower limit of quantization was 5 mg/ L.

PK parameters of SMX, including $AUC_{0\text{-}24h}$, volume of distribution (Vd), clearance (Cl) and half-life (t ½) were determined with a standard non-compartmental PK method using the KINFIT module of MW/Pharm 3.60 (Mediware, Groningen, The Netherlands). The $AUC_{0\text{-}24}$ in plasma was calculated according to the log –linear trapezoidal rule. In this case, the concentration of SMX at 24 hours after oral administration was estimated to be equal to its concentrations at zero time (before administration of the dose).

The free non-protein bound fraction of antibacterial drug is responsible for the clinical effect of these drugs [19]. The free $AUC_{0\text{-}24}$ (f $AUC_{0\text{-}24}$) values estimated by multiplying the total AUC0-24 values by the fraction unbound of 0.23 which was retrieved from an earlier study [19]. Free-drug AUC0-24/MIC ratios were calculated by dividing the f $AUC_{0\text{-}24}$ by the MIC value for SMX.

A one compartmental PK population model (POP-PK) of SMX with first order absorption without lag time was developed using an iterative two-stage Bayesian procedure (MW/ Pharm 3.60) starting with pharmacokinetic estimates from previous study [8].

The individual PK parameters of each patient were calculated using KINFIT. KINPOP is used to calculate the parameters of the MDR-TB patients based on the individual concentrations of SMX and patient characteristics like body weight, age, gender and creatinine clearance [20].

To determine the MIC values, the patients' *M. tuberculosis* isolates were subjected to DST which was performed on the Middelbrook 7H10 agar dilution method at the Dutch national Mycobacterium Reference Laboratory (National Institute for Public Health and the Environment, RIVM) [21]. In accordance with European Committee on Antimicrobial Susceptibility Testing, the MIC of SXT is expressed as TMP: SMX in the ratio 1: 19.

Safety

The safety of SXT during TB treatment was evaluated by assessing the reported side effects of SXT retrospectively using a standardized data abstraction form from the Tuberculosis Centre Beatrixoord. Specific attention was paid to the side effects that could be caused by SXT like gastrointestinal side effects (nausea, vomiting and diarrhea), hepatotoxicity, and anemia and blood count abnormalities [22].

Hepatic injury was defined as elevation in one of the hepatic enzymes five times the upper limit of normal during the treatment with SXT (grade 3 common toxicity criteria (CTC)). These enzymes include aspartate aminotransferase (ASAT> 200 $U.L^{-1}$), alanine aminotransferase (ALAT>225 $U.L^{-1}$) and gamma-glutamyl transpeptidase (GGT>200-275 $U.L^{-1}$) [23]. For other side effects the defined normal values were: anemia (hemoglobin normal range 7.5 – 9.9 mmol/L (female) and 8.7 – 10.6 mmol/L (male)), leukocyte count (4×10^9/L) and platelet count (150 - 350 $x10^9$ /L). The adverse drug reactions (ADR) in patients on multiple drug regimens like MDR-TB patients would require a standard causality assessment tool (Naranjo algorithm) [24]. Using this tool, we considered that SXT is definitely the cause of ADR if the score ≥ 9, probable if 5 to 8, possible if 1 to 4, and doubtful if the sore ≤ 0.

Statistics

Wilcoxon signed-rank test was employed in the statistical analysis when the data were not normally distributed. The POP-PK model was cross validated by developing a POP-PK model based on n-1 and by predicting the $AUC_{0\text{-}24h}$ of the subject left out during the model development. The correlation between the predicted based on the POP-PK model and calculated $AUC_{0\text{-}24h}$ was tested by means of a Bland and Altman analysis.

RESULTS

Patients

For 17 MDR-TB patients DST for SXT was performed. Only in ten patients SXT was used as part of their TB regimen because DST showed that isolates were susceptible to SXT. In the other cases resistance to SXT (n=4) or more conventional TB drugs could be used (n=3) (Table 2). In general, these TB patients were relative young with a median age of 29 years (Inter Quartile Range (IQR), 24-31years), and had a relatively low median body mass index (calculated as weight in kilograms divided by the square of height in meters) of 21.1(IQR, 19.1-23.6). The resistance of *Mycobacterium tuberculosis* to at least isoniazide and rifampicin was diagnosed by culture. Eight patients had Pulmonary TB as the most common diagnosis in MDR-TB patients, one patient had urogenital TB and one had both pulmonary and extra pulmonary TB. SXT was prescribed as 480 mg daily. This equals to a median dose of 6.5 mg/kg (IQR, 6.1-6.8) in various combination regimens for a median period of 381 (129-465) days. The daily dose of SXT was increased arbitrary to 960 mg which equals to 14 and 13 mg/kg in two patients because of low level of SXT in blood related to MIC in these patients. All of the patients had a negative history for underlying diseases except for one patient who had diabetes mellitus. None were diagnosed with co-infection with human immunodeficiency virus. Eight of the 10 patients successfully completed the treatment with no signs of recurrence. Two patients are still on treatment at the time of writing this report; sputum culture was converted and they are in good clinical conditions. The clinical data of all 10 patients are shown in Table 3. DST was evaluated in all MDR-TB patients. Susceptibility and resistance of *M. tuberculosis* against anti-TB drugs are shown in Table 2.

TABLE 2-Susceptibility and Resistance to anti TB drugs (n=17)

Group 1: First-line oral agents	**R**	**S**	**I**
Isoniazide	17 (100)		
Ethambutol	14 (82.4)	2 (11.8)	1 (5.9)
Rifampicin	17 (100)		
Pyrazinamide	9 (53)	8 (47)	
Rifabutine	13 (76.5)	3 (17.6)	1 (5.9)
Group 2: Injectable anti-TB-medication			
Amikacine	7 (41.2)	10 (58.8)	
Kanamycin	2 (11.8)	4 (23.5)	
Streptomycin	15 (88.2)	2 (11.8)	
Capreomycin	5 (29.4)	10 (58.8)	
Group 3: Fluoroquinolones			
Ofloxacin	1 (5.9)		
Moxifloxacin	3 (17.6)	13 (76.5)	1 (5.9)
Ciprofloxacin	4 (23.5)	12 (70.6)	
Group 4: Other bacteriostatic second line agents			
Protionamide	4 (23.5)	12 (70.6)	
Cycloserine	1 (5.9)	7 (41.2)	
Group 5: Antituberculosis drugs with unclear efficacy			
Linezolid	13 (76.5)	3 (17.6)	
Clofazimine		11 (64.7)	
Clarithromycin	2 (11.8)	5 (29.4)	
Augmentin	9 (53)	4 (23.5)	
Imipenem	2 (11.8)		
Others			
Co-trimoxazole	4 (23.5)	13 (76.5)	
Ertapenem		7 (41.2)	
Tigecycline	7 (41.2)	1 (5.9)	
Meropenem	1 (5.9)		

R: Resistance; S: susceptibility; I: intermediate susceptibility. Data are presented as absolute values (%) of MDR-TB patients susceptible, resistant and intermediately-susceptible to Anti-TB drug

TABLE 3- Characteristics of MDR-TB patients receiving SXT at baseline

Patient	sex	BMI (kg/m$^{2)}$	Duration of treatment (day) (SXT)	Total duration of treatment (day)	Ethnicity	co- morbidities and intoxications	Localization of TB	Other Anti- TB drugs
1	m	18.6	531	546	Hindustani	smoking, soft drugs	pulmonary	E,AM,MOX-,PTH, LZD, CFZ, Doxy
2	f	21.5	191#	202#	African	none	Pulmonary and extra pulmonary	MOX, KM, ERTA
3	m	22.1	501	705	Russian	alcohol abuse	pulmonary	Z, KM, LZD, CFZ, CLM, CM
4	f	18.1	412	548	Asian	none	pulmonary	E, AM, MOX, PTH, LZD, CFZ, CPX
5	f	19.3	154	191	Russian	none	pulmonary	MOX, LZD, AMX/CL, ERTA
6	m	20.7	350	365	Asian	smoking	pulmonary	E,AM,MOX, LZD, CFZ CPX ,Doxy
7	f	27.2	453	566	African	none	pulmonary	AM, MOX, CS, LZD
8	f	31	52	55	African	diabetes mellitus	extra pulmonary	AM, KM, MOX, LZD, CLM, ERTA
9	m	19.0	56	62	Russian	alcohol abuse, drug abuse, smoking	pulmonary	MOX, LZD, CLM, ERTA
10	m	21.1	44 #	41#	African	none	extra pulmonary	CLM, ERTA, LZD, KM

#Patients still on treatment. BMI: body surface area; m: male; f: female; Anti-TB; antituberculosis; E: ethambutol; SXT; Z: pyrazinamide; KM: kanamycin; AM: amikacin; CM: capreomycin; CPX: ciprofloxacin; CS: cycloserine; CLM; clarithromycin; MOX: moxifloxacin; CFZ: clofazimine; PTH: prothionamide; Doxy: doxycycline, ERTA: ertapenem; amoxicillin+clavulanic acid (Augmentin): AMX/CL; LZD: linezolid.

Pharmacokinetics (PK) and pharmacodynamics (PD)

The steady state PK parameters of SMX could be evaluated in only 8 of total 10 patients receiving 480 mg SXT once daily because in the other two patients no plasma sampling was performed during the treatment period with SXT. The PK parameters are summarized in Table 4. The observed plasma concentration- time curves of SMX were obtained from the patients after receiving 480 mg of SXT; these are shown in figure 1.

TABLE 4- Pharmacokinetic parameters of SMX at steady state after oral administration of 480 mg of SXT (n=8)

Pharmacokinetic parameter	SMX (400 mg)
AUC 0-24 (mg.h.liter^{-1})	371.5 (360-574.8)
Cl (ml/min/kg)	0.19 (0.14-0.25)
V (L/kg)	0.15 (0.13-0.22)
t ½ (h)	10.1 (8.7 -10.8)

Pharmacokinetic data are presented as median (interquartile range). AUC 0-24: area under the concentration-time curve up to 24 h post dosage; Cl: clearance; V: volume of distribution; t ½: half-life.

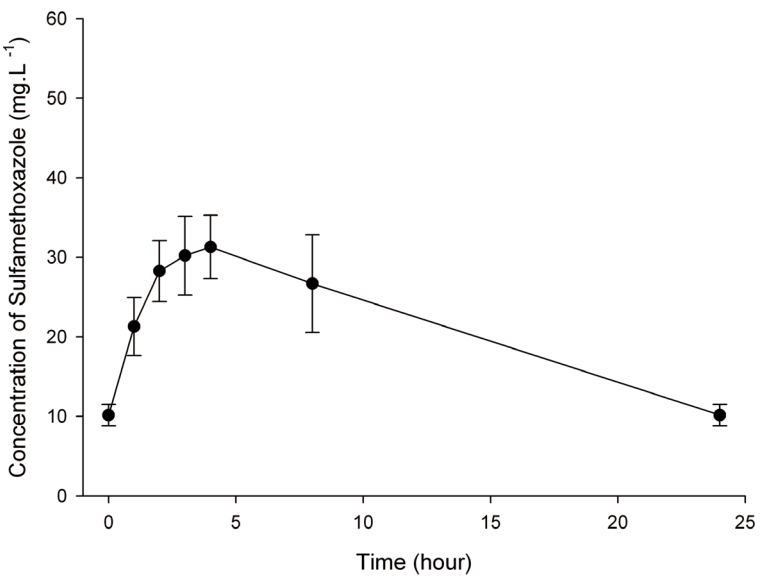

FIGURE 1-Plasma concentration-time curves of Sulfamethoxazole (n=8)

The parameters of POP-PK model of SMX are shown in Table 5. The cross validation of this model showed that the geometric mean values of the POP-PK model (n-1) were Cl (1.28 ± 0.52) L/h/1.85 m^2, Vd (0.22± 0.03) L/Kg LBMc, Ka-po (0.44±0.18), these results were not different from the POP-PK model (Table 5). The individual difference between the predicted based on POP-PK model and calculated values of AUC_{0-24} was underestimated by a median percentage of -0.7 (Range, -6.2-2.8). The agreement between predicted with the POP-PK and calculated AUC_{0-24h} of SMX is shown in figure 2. This figure shows that all the values of AUC were within the agreement only one was outside this agreement. The median percentage of difference between the predicted based on POP-PK (n-1) model and calculated values of AUC_{0-24} was -3.92 (Range, -6.3 -1.7).

The drug susceptibility testing shows that MICs values of SMX for *M. tuberculosis* varied with median ranges of 9.5 (IQR, 4.8-25) mg/L.

The ratios of $\int AUC_{0-24h}$/MIC of SMX in each patient are presented in Table 6. The geometric means of AUC/MIC and $\int AUC_{0-24h}$/MIC ratios after receiving 480 mg for SMX were 48.4 (IQR, 34.8-71.3) and 11.1 (IQR, 8 -16.4) respectively. One of the eight patients that received 480 mg of SXT had $\int AUC_{0-24h}$/MIC ratio of SMX greater than 25.

TABLE 5-Population pharmacokinetic model parameter values of SMX (n=8)

Parameter	Values
Cl (L.h/1.85 m^2)	1.14 ± 0.43
Vd (L. Kg^{-1} LbMc)	0.24 ± 0.05
Ka (h^{-1})	0.43 ± 0.17
F	1

Data are expressed as geometric mean ± standard deviation. Cl: apparent clearance; Vd: volume of distribution; Ka: absorption rate constant; F: bioavailability.

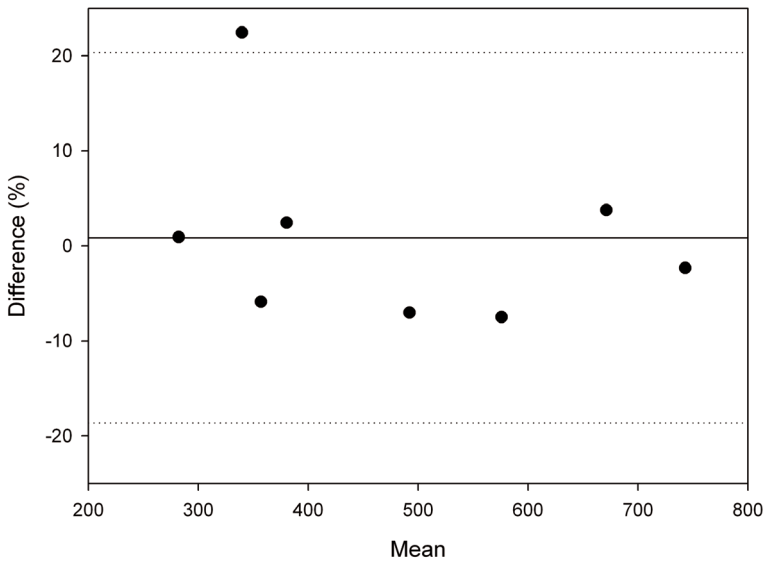

FIGURE 2-Bland-Altman plot showing the agreement between predicted and calculated area under the concentration-time curve up to 24 h post-dosage(AUC0-24) of Sulfamethoxazole

TABLE 6-*f*AUC0-24/MIC ratio of SMX after receiving 480 mg and 960 mg of SXT (n=8)

Patient no.	MIC (mg/L)	AUC_{0-24} (mg .h/L) 400 mg SMX	$fAUC_{0-24}$/MIC 400 mg SMX	AUC_{0-24} (mg .h/L) 800 mg SMX	$fAUC_{0-24}$/ MIC 800 mg SMX
1	25	376	3	774	7
2	4.75	297	14	-	-
5	9.5	658	16	991	24
6	25	509	5	-	-
7	4.75	597	29	-	-
8	4.75	367	18	-	-
9	19	752	9	-	-
10	4.75	281	14	-	-

AUC_{0-24}: area under the concentration-time curve up to 24 h post dosage; MIC: minimum inhibitory concentration; $fAUC_{0-24}$/MIC: protein unbound (free) AUC from 0 to 24 h relative to the minimal inhibitory concentration (MIC).

Safety

In general, SXT was well tolerated. However, there were some mild side effects including abdominal complaints with diarrhea and vomiting in one patient (Naranjo score=4). Elevations in hepatic enzymes (ASAT and ALAT) were observed in two patients that are receiving 480 and 960 mg of SXT respectively (Naranjo scores= 5). In these two patients the values of ALAT during treatment with 480 mg were 47.50 and 48 U.L -1 (baseline 29) and with 960 mg of SXT were 46 and 71 U.L -1 (baseline 43). The values of ASAT after receiving 480 and 960 mg of SXT were 48 and 99 $U.L^{-1}$ respectively compared with baseline 35. Although the hepatic enzymes were two times higher than base line, the values of ASAT and ALAT did not exceed 5 times the upper limit of normal. The median hemoglobin level in patients before treatment with 480 mg SXT was 7.6 mmol/L (range: 7.6 to 8 mmol/L) and during treatment was 7mmol/L (range: 6.6- to 7.2 mmol/L); the difference was significant (P=0.002) but probably, clinically not relevant. The value of Naranjo score for low hemoglobin level is 3 or 4. One patient developed leucocytopenia after receiving 480 and 960 mg of SXT. Leucocyte counts were 2.2×10^9/L and 3.2×10^9/L respectively in comparison to a baseline value of 4.2 (Naranjo score=3). Besides, one patient developed mild thrombocytopenia with a thrombocyte count of 146 $x10^9$ /L and of 247 at baseline (Naranjo score = 3).

DISCUSSION

No earlier study described the PK, PD and PK/PD parameters of SXT in MDR-TB patients. It is interesting is that the PK parameters of SMX in TB patients including $AUC_{0\text{-}24h}$, Vd and Cl are lower than the values observed in patients with meningitis, HIV infections or those who suffering from bacterial skin infection (Table 1) [6-9]. Low drug exposure may be explained by decreased intestinal absorption resulting in low serum concentrations of antituberculous drugs. Other factors such as alcohol abuse, smoking, weight loss, albumin and hemoglobin could be the possible reasons for reduced permeability via paracellular intestinal transport [25]. To further explore the PK parameters in MDR-TB patients, we made a population model. This model showed no significant difference between the calculated and predicted AUC according to Bland-Altman analysis and this difference was also statistically insignificant *(P*=0.78). Thus, MDR-TB patients seem to display a consistent PK profile for SMX. Therefore, this developed model could be used to assess drug exposure in a prospective study to evaluate the safety and efficacy and find the suitable dose of SXT as part of a TB-regimen.

The MIC value of SMX in this study was in accordance with previous study that mentioned that SMX inhibits 80% growth of all 117 isolates at an MIC 19 mg/L [10].

The PK-PD parameter ($AUC_{0\text{-}24}$/MIC) best predicting of SMX efficacy has not been firmly established. According to an earlier single study, the f $AUC_{0\text{-}24}$/MIC of SMX had to exceed 25 for adequate treatment of melioidiosis [12]. From the results, it can be seen that only in 1 of 8 patients, f $AUC_{0\text{-}24}$/MIC ratio of SMX was > 25. However, this ratio could be lower than 25 and still be effective in TB treatment. Especially the magnitude of this parameter may vary for different bacterial species. For example, the $AUC_{0\text{-}24}$/MIC ratio for fluoroquinolone is different against *Pseudomonas aeruginosa* and other gram-negative bacilli [26]. The lower ratio might be acceptable in patients receiving multidrug treatment for MDR-TB. Other drugs might decrease this ratio as shown in a murine aerosol infection model from previous study in which the $AUC_{0\text{-}24}$/MIC ratio of rifampicin that correlated with efficacy decreased when administered in combination with moxifloxacine [27]. Therefore, the interpretation of the value of the $AUC_{0\text{-}24}$/MIC for SMX in our MDR-TB-patients at this time is difficult. The dose of SXT given to the MDR-TB patients was low compared to commonly used dosages for other infectious diseases. However, one has to keep in mind that the more conventional dosages of SXT are for the treatment of fast replicating bacteria and is often given as mono therapy. In our case the aim is slow growing *M. tuberculosis* in combination with other antimicrobial agents. For future study of SXT for MDR-TB it's can be advised to also explore higher dosages to achieve higher drug exposure but tolerability may be a problem during prolonged treatment.

The TB patients with HIV infections can have drug–drug interaction (DDI) when rifampin (RIF) is co-administered with SXT as mentioned in previous study [8] but this is of no concern in MDR-TB. Indeed, RIF decreases the concentrations of TMP and SMX significantly in serum but again, SXT would not be prescribed in individuals that can be treated with RIF and non-received RIF before starting the treatment with SXT.

Although our sample size was low, this retrospective study confirmed the safety of SXT in accordance with earlier studies that showed that SXT was safe and well tolerated when it was used as prophylaxis in adults with HIV infection who have pulmonary TB [28, 29]. SXT was well tolerated in MDR-TB patients and was not discontinued in any of 8 patients till the end of treatment and in 2 patients that are still on treatment. Only one patient has gastrointestinal complaints as a possible side effect of SXT following administration of 960 mg of SXT daily. Therefore, the dose was lowered to 480 mg till the end of treatment. In our patients, the maximum Naranjo score of 5 was reached, in other words there is a probable relationship between the observed side effects and SXT. However hematological side effects including anemia, leucocytopenia and thrombocytopenia during treatment could be due to other antituberculosis drugs like linezolid [30, 31].

The main limitation of this study is that there is a lack of data on the target (AUC/MIC value) to be reached to predict the efficacy of SXT in the treatment of TB. In the future, a

prospective study is needed to evaluate the PK and PD parameters of SXT in TB-patients. To determine $fAUC_{0\text{-}24}$/MIC ratio of SMX for effective treatment along with suppression of the emergence of drug resistance, an *in vitro* infection model could be the suitable strategy as reported previously for moxifloxacin [32].

Comparing SXT with other drugs not registered for TB treatment but used in MDR-TB regimen, it has the great advantage that it is cheap and readily available all over the world.

These are the first results on the inclusion of SXT in MDR-TB treatment in which drug susceptibility testing and SXT concentration measurements were combined. Based on our preliminary data we showed that SXT has a favorable PK profile in TB patients.

Further in vitro PK and PD studies like a hollow fiber infection model or mouse model is warranted to establish target AUC/MIC value to predict the efficacy of SXT and consequently to set the optimal dose in the treatment of MDR-TB treatment. Preferably, PK/PD parameters of SXT, MIC of SXT should be measured alone and in the presence of other antituberculous drugs to detect the possible synergism between these drugs. According to the clinical outcome that showed no treatment discontinuation or serious side effects, the consistent PK values and relative low MIC values, this study could be the starting point for further exploration of SXT for MDR-TB treatment.

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CHAPTER 3

TRIMETHOPRIM/SULFAMETHOXAZOLE SUSCEPTIBILITY OF MYCOBACTERIUM TUBERCULOSIS

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Trimethoprim/sulfamethoxazole susceptibility of Mycobacterium tuberculosis

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Tuberculosis (TB) is one of the major infectious diseases in the world. TB treatment has become increasingly complicated because of co-infection with HIV and the emergence of resistant *M. tuberculosis* strains [1]. This stresses an urgent need for alternative, effective drugs with a minimum of toxicity. An old, inexpensive and well-tolerated drug like co-trimoxazole (SXT) could be an attractive option for the treatment of MDR and XDR-TB [2].

SXT has been widely prescribed as prophylaxis for *Pneumocystis jirovecii* pneumonia (PCP) in human immunodeficiency virus (HIV)-infected persons, and has been associated with a significantly decreased risk for other opportunistic infections. Limited data are available on the *in vitro* SXT susceptibility of *M. tuberculosis,* but it appears that only SMX is effective against *M. tuberculosis* while TMP is not [2, 3].

The objective of this study was to evaluate the susceptibility of *M. tuberculosis* strains from normal sensitive TB and HIV-TB patients to SMX, and to compare these results with MICs values of SMX from MDR-TB patients that received SXT as part of their MDR-TB treatment in our previous retrospective study [4].

A total of 50 *M. tuberculosis* isolates were selected, 15 of which were normal sensitive TB; 18 were from HIV/TB patients, of which 13 received SXT and five did not receive SXT as PCP prophylaxis during their treatment; and 17 from MDR-TB HIV-negative patients from the strain collection of the Tuberculosis Reference Laboratory at the National Institute for Public Health and the Environment (RIVM) in Bilthoven, the Netherlands.

The susceptibility of the respective *M. tuberculosis* strains to SXT was determined according to the Absolute Concentration Method. *M. tuberculosis* susceptibilities to SXT from HIV-TB patients were measured at baseline, prior to the start of TB treatment. For this study, *M. tuberculosis* was defined as susceptible to SXT when the MIC breakpoint is ≤ (1-2) mg/L, and resistant to SXT when the MIC breakpoint is (> 2 - ≥4) mg/L comparable to breakpoints of other bacteria.

MIC values were not normally distributed. The Mann–Whitney *U*-test was used to compare the MIC of MDR-TB, HIV-TB and drug-sensitive TB patients. Values are median ± interquartile range (IRQ). A P-value of <0.05 was considered significant.

MICs of SXT in clinical isolates of *M. tuberculosis* from patients with drug-susceptible TB, with or without HIV co-infection, and patients with MDR-TB were determined (Table1). The MIC of SXT is expressed as MIC of TMP, and the MIC ratio of TMP to SMX is 1:19.

Resistance of *M. tuberculosis* isolates from HIV-TB patients was only seen in one of the 13 patients that received SXT as prophylaxis. Of the 13 HIV-TB patients that received SXT, only one did before TB treatment for five months. In the remaining 12 HIV-TB patients SXT was started with other anti-TB drugs for a median treatment period of 23 days (Interquartile range 9-105). No significant difference was observed between the MICs of

the isolates from drug-susceptible TB patients with either HIV/TB confection (P=0.927) or MDR-TB (P=0.168). Thirteen of 17 MDR-TB isolates tested were susceptible to SXT, and the remaining four MDR-TB isolates were SXT-resistant. Although this study showed that resistance of *M. tuberculosis* to SXT is limited in HIV-TB and MDR-TB patients, the number of strains was probably insufficient to detect the emergence of resistance.

We speculated that SXT given to HIV-TB patients in combination with first-line treatment could have beneficial effects on the outcome of TB treatment, considering the susceptibility of *M. tuberculosis* isolates from HIV-TB patients and a synergistic effect of SMX and rifampicin plus an additional effect with ethambutol [5].

The MICs of SMX reported in these isolates are remarkably similar to the MICs reported in a previous study [3]. In a small cohort study, SXT showed favourable drug exposure, a low MIC in the *M. tuberculosis* isolates tested, and only limited toxicity [4]. This suggests that SXT could be of value in the treatment of MDR-TB in the future.

Given that only a single patient received SXT prophylaxis before TB was diagnosed, our study was not designed to detect emergence of SXT resistance during treatment, which has been observed previously during long-term prophylaxis with SXT [6]. The other patients started prophylaxis with SXT at the time of TB diagnosis and HIV co-infection. In other clinical settings HIV may be diagnosed earlier than TB. Patients may receive SXT as a single drug long before TB is diagnosed and SXT resistance may be induced. For this reason, the emergence of resistance to SXT should be closely monitored and evaluated in areas of the world where the TB and HIV epidemics overlap.

In conclusion: because of the SXT susceptibility of *M. tuberculosis* isolates from HIV-TB and MDR-TB patients, SXT was found promising for further exploration in the treatment of HIV-TB and MDR-TB patients.

TABLE1- Minimum inhibitory concentrations (MICs) for 50 isolates of Mycobacterium tuberculosis to trimethoprim/sulfamethoxazole

Clinical isolate	MIC(mg/L) [a]		
	Drug-susceptible TB, HIV-negative patients(n=15)	Drug-susceptible TB, HIV-positive patients(n=18)	MDR-TB, HIV-negative patients(n=17)
1	0.5	<0.25	>2
2	1	[b] 0.5	<0.25
3	0.5	0.5	0.5
4	1	0.5	0.5
5	<0.25	4	0.5
6	1	0.5	0.5
7	0.25	0.5	>2
8	<0.25	0.5	>4
9	<0.25	1	2
10	0.5	<0.25	<0.25
11	0.5	0.5	0.5
12	0.5	<0.25	4
13	0.5	0.5	<0.25
14	0.5	0.5	<0.25
15	0.25	<0.25	1
16		0.5	1
17		0.5	<0.25
18		0.5	
Median(IRQ) MIC	0.5 (0.25-5)	0.5 (0.5-0.5)	0.5 (0.25-2)
MIC< 2mg/l(n)	15	17	13
MIC > 2mg/l (n)	-	1	4

TB, tuberculosis; HIV, human immunodeficiency virus; MDR, multidrug-resistant; IQR, interquartile range.
[a] The MIC of SXT is expressed as the MIC of trimethoprim (TMP), and the MIC ratio of TMP to SMX is 1:19.
[b] MIC of only one HIV/TB-positive patient who received SXT before TB treatment for 5 months

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CHAPTER 4

QUANTIFICATION OF CO-TRIMOXAZOLE IN SERUM AND PLASMA USING TANDEM MASS SPECTROMETRY

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Quantification of Co-trimoxazole in serum and plasma using tandem mass spectrometry

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ABSTRACT

Background

Co-trimoxazole is frequently used in the prophylaxis and treatment of pneumocystis carinii pneumonia. High plasma concentrations of sulfamethoxazole or trimethoprim are correlated with toxicity. There is, however, a large variation in pharmacokinetics observed which can lead to underexposure or toxicity.

Results

We developed a novel liquid chromatography tandem mass spectroscopy (LC/MS-MS) method to analyse the components of co-trimoxazole, trimethoprim and sulfamethoxazole and its metabolite Sulfamethoxazole-N-acetyl. This new method is expeditious due to its limited sample pre-processing and a relatively short run-time of only 3 minutes.

Conclusion

This new method met the FDA requirements on linearity, selectivity, precision, accuracy, matrix effects, recovery and stability and is suitable for routine analysis and future prospective studies.

INTRODUCTION

Co-trimoxazole is a cheap and effective drug for the treatment and prophylaxis of pneumocystis carinii pneumonia (PCP) in HIV patients [1-3]. Improved survival was observed after initiation of combination antiretroviral therapy (cART) accompanied by co-trimoxazole treatment in HIV-infected patients [4]. According to the World Health Organization (WHO), co-trimoxazole should be continued until full recovery of the immune system [5].

A recent prospective cohort study in Switzerland showed that co-trimoxazole prophylaxis against PCP in the treatment of HIV reduced the incidence of tuberculosis [6]. Based on observations like this one and the in vitro activity of sulfamethoxazole against Mycobacterium tuberculosis [7-9] it has been suggested that sulfamethoxazole may be useful in the treatment of tuberculosis. In TB-HIV co-infected patients it was shown that trimethoprim and sulfamethoxazole drug exposure were highly variable between individuals [10, 11].

Well-known adverse events like neutropenia and thrombopenia were associated with increased serum concentrations of co-trimoxazole [10-12]. Furthermore, sulfamethoxazole is metabolized into Sulfamethoxazole-N-acetyl by N-acetyltransferase. This metabolite is less soluble than sulfamethoxazole, and is known to cause crystalluria resulting in obstruction and renal damage [13]. Blood concentration measurements of sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim and subsequent dose adjustments could possibly reduce these side effects.

Although co-trimoxazole has been available for many years the PK/PD target of has not been elucidated yet. The effect of sulfamethoxazole seems to depend on the area under the curve (AUC) divided by the MIC, and the time above MIC (T>MIC) [14]. Sulfamethoxazole was effective in the treatment of melioidosis, which is caused by Bukholderia pseudomallei, with a T>MIC of 60% [14]. However, the validity of this PK/PD target in the treatment in other infectious diseases, such as PCP and possibly tuberculosis, is unclear.

To be able to further explore the relation between co-trimoxazole drug concentration and efficacy and toxicity a suitable analytical method is required. Therefore, the objective of this study was to develop a simple, reliable and robust LC-MS/MS method to measure concentrations of trimethoprim, sulfamethoxazole and sulfamethoxazole-N-acetyl in human serum and plasma.

METHODS

Experimental

Sulfamethoxazole, trimethoprim and trimethoprim-D9 were obtained from Sigma Aldrich (MO, USA). Sulfamethoxazole-N-acetyl and its internal standard sulfamethoxazole-N-acetyl-D4 were purchased from Santa Cruz (TX, USA). Sulfamethoxazole-D4 was obtained from Alsachim (Illkirch Graffenstaden, France). The chemical structures of sulfamethoxazole, sulfamethoxazole-D4, Sulfamethoxazole-N-acetyl, Sulfamethoxazole-N-acetyl-D4, trimethoprim and trimethoprim-D9 are shown in figure 1. Ammonium acetate and acetic acid were retrieved from Merck (NJ, USA). Trifluoroacetic acid and acetonitrile LC-MS were both obtained from Biosolve (Dieuze, France). Water was in house purified using a Milli-Q system (Millipore Corporation, MA, USA).

Sulfamethoxazole-D4 was used as internal standard to quantify sulfamethoxazole. Sulfamethoxazole-N-acetyl was quantified using Sulfamethoxazole-N-acetyl-D4 and trimethoprim-D9 was used as internal standard for the trimethoprim quantification. The internal standard solution contained 200 ng/mL sulfamethoxazole-D4, 2,000 ng/mL Sulfamethoxazole-N-acetyl-D4 and 10 ng/mL trimethoprim-D9 dissolved in methanol (Merck, NJ, USA).

Buffer solution used in the gradient elution consisted of ammonium acetate (5.0 g/L), acetic acid (100%, 35 mL/L) and trifluoroacetic acid (100%, 2 mL/L).

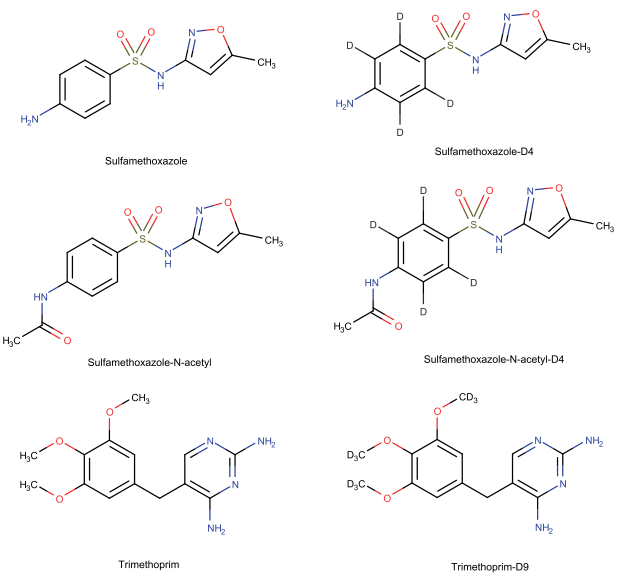

FIGURE 1 -Structures of sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim and their respectively internal standards sulfamethoxazole-D4, Sulfamethoxazole-N-acetyl-D4 and trimethoprim-D9.

Analysis

A TSQ Quantum Access Max (TSQ Quantum, Thermo Scientific, San Jose, CA, USA), supplied with a Finnigan Surveyor MS Pump Plus and a Finnigan Surveyor Autosampler Plus was used to perform the analysis. The mass spectrometer was equipped with a Thermo Scientific Hypurity Aquastar C18 (50*2.1 mm) column with a particle size of 5 µm. The spray voltage was set to 3500V. Sheath and auxiliary gas pressure were set on 35 and 10 bar, respectively. Capillary temperature was set on 350 °C. Autosampler temperature was set to 10 °C.

The following ion transitions were selected: 254.0 → 156.1 (sulfamethoxazole), 258.1 → 160.1 (sulfamethoxazole-D4), 296.1 → 198.0 (Sulfamethoxazole-N-acetyl), 300.1 → 202.1 (Sulfamethoxazole-N-acetyl-D4), 291.1 → 230.0 (trimethoprim, figure 2a), 300.2 → 234.1 (trimethoprim-D9, figure 2b).

Gradient elution was used as displayed in table 1. A continuous flow of 500 µl/min was used. Observed retention times were 1.75, 2.06 and 1.52 min for sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim, respectively. The internal standards sulfamethoxazole-D4, Sulfamethoxazole-N-acetyl-D4 and trimethoprim-D9 eluted at 1.73, 2.05 and 1.49 minutes after injection, respectively.

TABLE 1 - Gradient elution

Time (min)	A (%)	B (%)	C (%)
0.00	5	90	5
0.40	5	90	5
0.40	5	77.5	17.5
2.00	5	70	25
2.01	5	0	95
2.60	5	0	95
2.61	5	90	5
3.00	5	90	5

A: ammonium acetate 5.0 g/L, acetic acid 100% 35 ml/L, trifluoroacetic acid 2 mL/L. B: ultra pure water, C: acetonitrile LC-MS

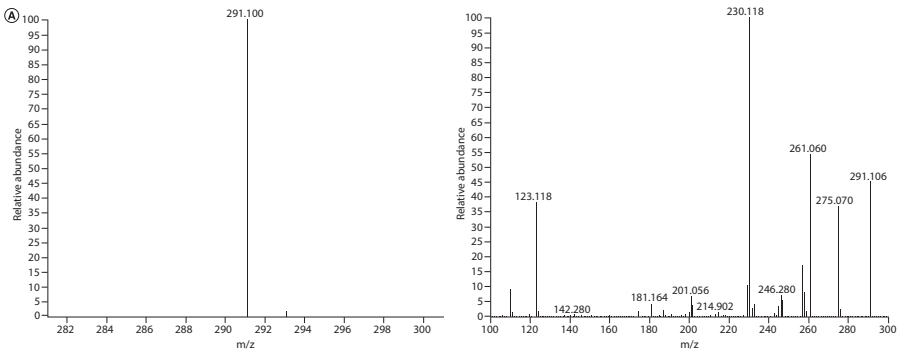

FIGURE 2A - Trimethoprim product ion scan

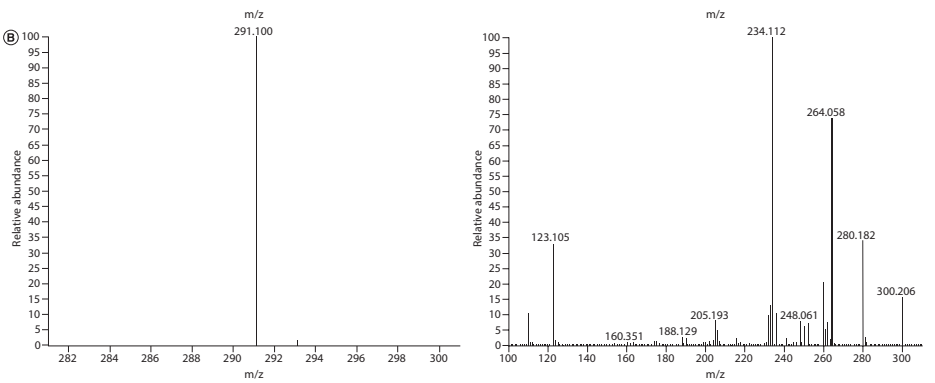

FIGURE 2B - Trimethoprim-d9 product ion scan

Sample preparation

From the serum or plasma sample, 10 µL was transferred into an Eppendorf tube. After the addition of 250 µL of the internal standard solution, the sample was vortexed for 1 minute. The vortexed sample was centrifuged for 5 minutes at 10,000 rpm and 200 µL of the extract with 500 µL ultrapure water was transferred into the vial. The vial was vortexed for 1 minute and was subsequently ready for analysis.

Analytical method validation

Validation is carried out in concordance with Food and Drug Administration (FDA)

guidelines [15] in human serum. Tests for selectivity, accuracy and precision, stability, recovery and matrix effects were carried out using LOW, MED and HIGH quality control (QC) samples. The concentration of the QC samples is displayed in table 2.

Selectivity for all three analytes was determined by analysing six separate serum samples, each from a different serum pool. Ion suppression was tested using a post-column infusion test [16].

The calibration curve of trimethoprim consisted of 8 concentration levels: 200, 500, 1,000, 1,500, 2,500, 5,000, 7,500 and 10,000 ng/ml. Calibration curves of both sulfamethoxazole and Sulfamethoxazole-N-Acetyl consisted also of 8 concentration curves: 2,000, 5,000, 10,000, 15,000, 25,000, 50,000, 75,000 and 100,000 ng/ml. The regression formulas were calculated using One-Way ANOVA. Linearity was tested with a Lack-of-fit sum of squares F-test.

Human serum was spiked with all QC concentrations separately and analysed in five replicates on three consecutive days. The accuracy was determined by calculating the difference of all measurements versus the nominal value for each QC concentration level of all three days combined (n = 15 per QC level). The within-day and between-day precision was calculated using the coefficient of variation (%) for each QC concentration level.

Stability was evaluated by spiking blank serum with LOW and HIGH QC concentrations of all three analytes. Bench-top stability was assessed after 240 hours at room temperature. Post-extraction stability was determined after 120 hours in the auto sampler at 10 °C. Freeze-thaw stability was determined after 5 cycles of freezing (at -20 °C) and thawing (at room temperature). The stability was calculated by comparing the test samples with freshly prepared calibration standards.

Dilution integrity of sulfamethoxazole and Sulfamethoxazole-N-acetyl was determined by diluting a 200,000 ng/mL serum sample to 20,000 ng/mL in five replicates on three different days with blank serum. For trimethoprim, dilution integrity was tested by diluting a 20,000 ng/mL solution to 2,000 ng/mL with blank serum.

Blank serum was spiked with three QC concentration levels (LOW, MED and HIGH). The peak area of the peaks was compared to spiked extraction fluid to determine the matrix effect [17]. The recovery was calculated by dividing the peak area of spiked serum by the peak area of spiked extracted blank serum, again at three concentration levels (LOW, MED and HIGH).

Matrix comparison tests were performed to compare the effect of human serum and human plasma on the analytical outcome. The calibration lines, in serum and plasma, were considered comparable when the 95% confidence interval of the intercept and slope were non-significantly different.

Table 2 - Validation results

Criteria	QC concentration level			
	LLOQ	LOW	MED	HIGH
Nominal concentration (ng/mL)				
Sulfamethoxazole	2,000	10,000	40,000	80,000
Sulfamethoxazole-N-acetyl	2,000	10,000	40,000	80,000
Trimethoprim	200	1,000	4,000	8,000
Accuracy (bias (%))				
Sulfamethoxazole	-2.8	-4.4	0.5	0.2
Sulfamethoxazole-N-acetyl	-5.5	-7.2	-1.9	-2.6
Trimethoprim	-1.9	-3.9	1.2	3.8
Within-day precision (CV (%))				
Sulfamethoxazole	5.0	2.5	3.9	3.0
Sulfamethoxazole-N-acetyl	12.9	9.1	5.6	4.6
Trimethoprim	4.3	2.3	3.4	2.7
Between-day precision (CV (%))				
Sulfamethoxazole	0.0	1.4	0.0	0.0
Sulfamethoxazole-N-acetyl	0.0	1.0	4.3	3.3
Trimethoprim	3.4	2.3	2.6	3.1
Recovery (%)				
Sulfamethoxazole	n.d.	93.1	88.8	97.9
Sulfamethoxazole-N-acetyl	n.d.	94.2	86.9	105
Trimethoprim	n.d.	93.7	90.1	98.3
Matrix effect (%)				
Sulfamethoxazole	n.d.	106.4	100.7	103.6
Sulfamethoxazole-N-acetyl	n.d.	111.5	102.4	96
Trimethoprim	n.d.	107.6	102.3	103.6
Autosampler stability (120h bias %)				
Sulfamethoxazole	n.d.	-6.2	n.d.	0.6
Sulfamethoxazole-N-acetyl	n.d.	-13.7	n.d.	-2.9
Trimethoprim	n.d.	-7.4	n.d.	4.1
Bench top stability (240h) (bias %)				
Sulfamethoxazole	n.d.	0.3	n.d.	6.7
Sulfamethoxazole-N-acetyl	n.d.	-0.4	n.d.	6.8
Trimethoprim	n.d.	3.8	n.d.	11.1
Freeze-thaw stability (after five freeze-thaw cycles) (bias %)				
Sulfamethoxazole	n.d.	-4.7	n.d.	1.6
Sulfamethoxazole-N-acetyl	n.d.	-7.4	n.d.	-4.3
Trimethoprim	n.d.	-1.6	n.d.	6.3

n.d.: not done

RESULTS

Analytical method validation

The chromatogram of the LOW concentrations of sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim is shown in figure 3. No peaks were observed in the chromatogram on the retention times of all three compounds in extracted blank serum and plasma. No ion suppression was observed.

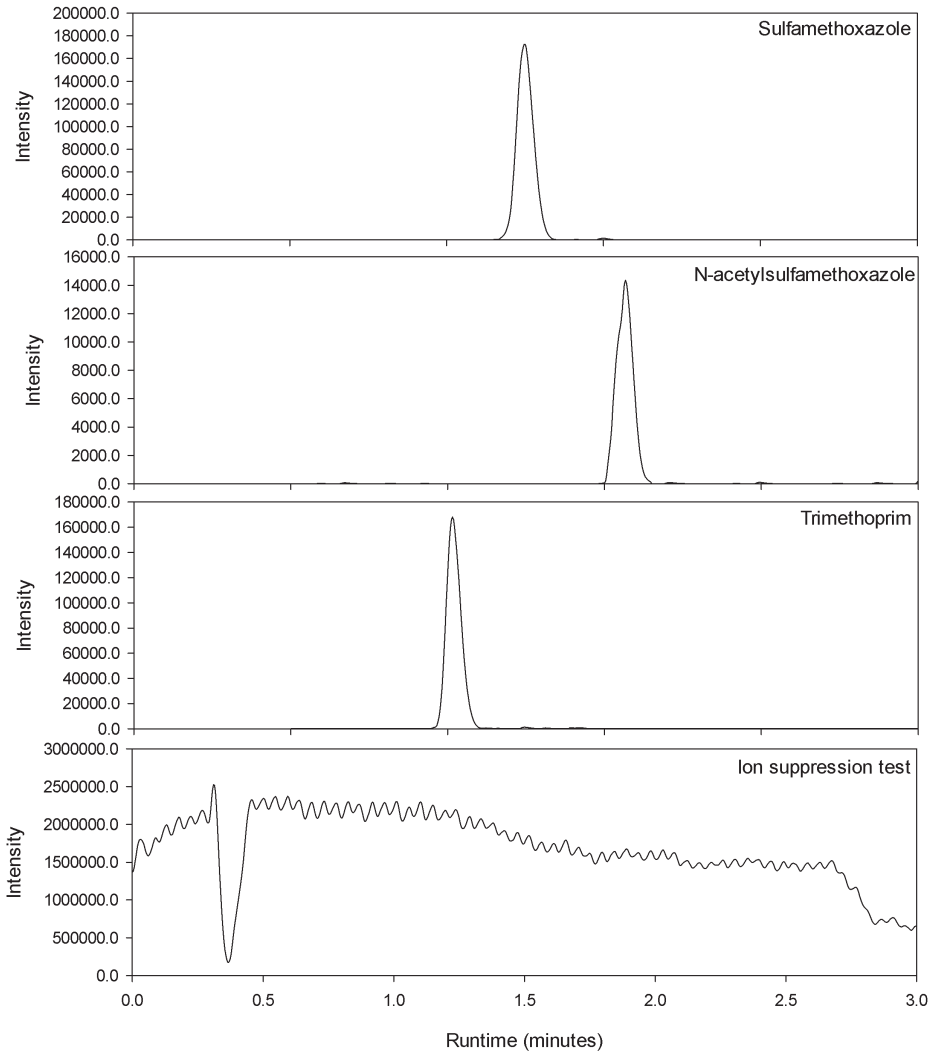

FIGURE 3 - Chromatogram of the LOW QC concentrations in extracted samples of sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim.

The properties of the calibration curves are displayed in table 3. All lines showed a correlation and regression coefficient of 0.99 or higher.

Table 3 -Calibration curves

Compound	Y-intercept (± st. dev)	Slope (± st. dev)	Correlation coefficient	Regression coefficient
Sulfamethoxazole	0.0231 ± 0.0136	0.274 ± 0.00266	0.999	0.998
Sulfamethoxazole-N-acetyl	-0.0173 ± 0.00586	0.0730 ± 0.00115	0.997	0.995
Trimethoprim	-0.0149 ± 0.0130	1.51 ± 0.0254	0.997	0.994

The accuracy of sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim varied from -4.4 – 0.5%, -7.2 – -1.9% and -3.9 – 3.8%, respectively. Within-day precision (CV (%)) ranged from 2.3 – 12.9% for all three analytes at all QC concentration levels. Between-day precision (CV (%)) was determined and varied from 0.0 – 4.3%.

The bench top stability was evaluated; the bias in concentration of all compounds was calculated at ≤ 11.1%. The bias found in the post-extraction stability test varied between -13.7 – 4.1%. Freeze-thaw stability tests showed a bias of -7.4 – 6.3%, as shown in table 2.

For sulfamethoxazole and Sulfamethoxazole-N-acetyl, matrix effects biased the analytical outcome with 0.7 – 6.4% and -4.0 – 11.5% respectively. A difference of 2.3 – 7.6% was found for trimethoprim. Recoveries varied between 88.8 – 97.9% for sulfamethoxazole, 86.9 – 105.0% for Sulfamethoxazole-N-acetyl and 90.1 – 98.3% for trimethoprim.

No statistical difference in slope and Y-intercept of the calibration line in serum and plasma was found for all three analytes sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim. The matrix comparison curves are displayed in figure 4.

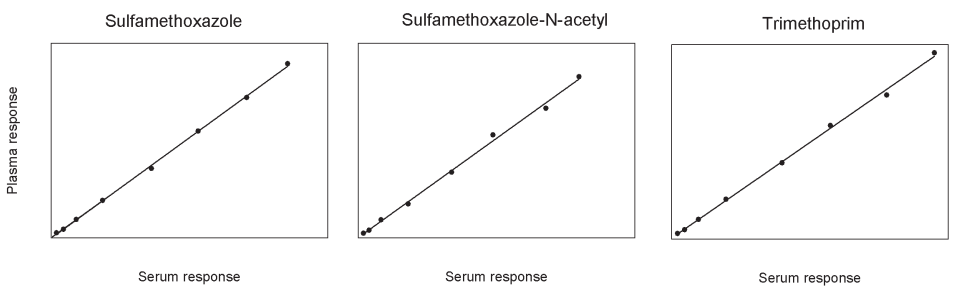

FIGURE 4 -Matrix comparison of sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim in human serum and plasma

DISCUSSION

We developed a novel robust LC-MS/MS assay to determine sulfamethoxazole, Sulfamethoxazole-N-acetyl and trimethoprim simultaneously in plasma or serum. This method is validated based on the FDA guidelines on precision, bias, recovery, matrix effect, stability and dilution integrity. This new method can be applied in pharmacokinetic studies of co-trimoxazole in a range of infectious diseases.

The LC-MS/MS method described in this paper has several advantages. All three compounds are quantified based on their corresponding deuterated internal standard, which is structurally highly similar to the analyte. In order to reduce costs, the concentrations of the internal standards are kept relatively low which resulted in internal standard responses comparable to LLOQ or LOW QC responses for the corresponding analytes. However, the response of the internal standard was sufficient to reliable quantify all analytes. Also, the method is validated for a broad concentration range and requires only a small amount of blood, which makes it also suitable for quantification of trimethoprim, sulfamethoxazole and its metabolite in paediatric studies.

A major problem during the development of the method was the separation of sulfamethoxazole and Sulfamethoxazole-N-acetyl with the LC system within the short run time, probably due to the similar structure and polarity of both compounds. We resolved this issue by adding ultrapure water to the sample mixture, which improved the separation within three minutes' run time. This separation was further enhanced by optimizing the gradient elution.

Papers describing the analysis of co-trimoxazole in serum with HPLC-UV are already available [10, 18, 19]. However, the use of LC-MS/MS has several advantages above HPLC-UV, such as a higher specificity, sensitivity and an easier sample preparation. Only three papers could be retrieved addressing the validation of the analysis of co-trimoxazole in serum using LC-MS/MS [20-22]. Unfortunately, all three papers did not include the validation of Sulfamethoxazole-N-acetyl, the toxic metabolite of sulfamethoxazole. Furthermore, one paper used solid phase extraction (SPE), which is time-consuming and results in additional costs of consumables and a longer turn-around time. Also, trimethoprim was validated to 5,000 ng/mL, which is insufficient for TDM in the treatment of PCP where higher serum concentrations are observed [12]. Additional sample dilution will be needed in these cases. These limitations make this method not ideal for TDM. Our method is able to analyse Sulfamethoxazole-N-acetyl and uses a simplified sample work-up, minimizing the time needed for analysis. In addition, the quantification of trimethoprim is validated to 10,000 ng/mL, which should be sufficient to measure trimethoprim levels during PCP treatment.

This new method makes it possibly to quantify co-trimoxazole and its toxic metabolite in serum and plasma in a reliable, efficient and robust way. This method can be used to further study the pharmacokinetics and pharmacodynamics in a range of infectious diseases in order to optimize treatment.

CONCLUSION

The new developed method proved to provide a reliable and robust quantification of sulfamethoxazole, its toxic metabolite Sulfamethoxazole-N-acetyl and trimethoprim in serum and plasma suitable for TDM and clinical studies.

Executive summary

Introduction

Co-trimoxazole is used for many infectious diseases, such as Pneumocystis carinii pneumonia.

In the era of the emergence of drug resistance, old antibiotics are re-evaluated for their potential against new threats.

Sulfamethoxazole, one out of two components of co-trimoxazole, may also be effective against multidrug resistant tuberculosis.

The pharmacokinetics of sulfamethoxazole are highly variable between individuals, which urges the need for dose adaptation guided by the blood concentration.

Experimental

A novel and robust liquid chromatography tandem mass spectrometry method to quantify trimethoprim, sulfamethoxazole and its nephrotoxic metabolite sulfamethoxazole-N-acetyl was developed.

This new method requires only limited sample pre-processing without solid phase extraction and is able to separate all three analytes in a short chromatography runtime of 3 minutes.

Results

This new method was validated based on the FDA guidelines on selectivity, accuracy, precision, recovery, matrix effect and stability.

A matrix comparison in serum and plasma was performed to confirm that both matrices were suitable for quantification.

Discussion

With this new method, the blood concentrations of trimethoprim and sulfamethoxazole can be measured for daily practice and future prospective studies.

FUTURE PERSPECTIVE

Drug resistance of various microbes is emerging. The activity of old antibiotics, such as co-trimoxazole, against various microbes should therefore be re-evaluated. Sulfamethoxazole, one out of two components of co-trimoxazole, may be effective against multidrug resistant tuberculosis. With our novel method of analysis, new prospective research could be done to find the most optimal pharmacokinetic/pharmacodynamics (PK/PD) parameter of sulfamethoxazole in the treatment of tuberculosis.

With this PK/PD data, new regimens incorporating co-trimoxazole can be designed in the treatment of multidrug and extensively drug resistant tuberculosis. This to ultimately reduce the treatment duration needed to treat multidrug resistant tuberculosis and to generate treatment possibilities in the case of extensively resistant tuberculosis.

ACKNOWLEDGEMENTS: none to declare

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CHAPTER 5

A PHARMACOKINETIC EVALUATION OF SULFAMETHOXAZOLE 800 MG ONCE DAILY IN THE TREATMENT OF TUBERCULOSIS

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A pharmacokinetic evaluation of sulfamethoxazole 800 mg once daily in the treatment of tuberculosis

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ABSTRACT

For treatment of multi drug resistant tuberculosis (MDR-TB) there is a scarcity of antituberculosis drugs. Co-trimoxazole is one of the available drug candidates, and already frequently co-prescribed in TB-HIV co-infected patients. However, only limited data are available on pharmacokinetic (PK) and pharmacodynamic (PD) parameters of co-trimoxazole in TB patients. The objective of this study was to evaluate PK parameters and *in* vitro PD data of the effective part of co-trimoxazole; sulfamethoxazole. In a prospective PK study in patients with drug-susceptible TB (age >18), SXT was administered orally in a dose of 960 mg once daily. One-compartment population pharmacokinetic modelling was performed using Mw\Pharm 3.81 (Mediware, Groningen, The Netherlands). The *ƒ*AUC/MIC ratio and the time period in which the free concentration exceeded the MIC (T>MIC) were calculated. Twelve patients received 960 mg co-trimoxazole on top of first line drugs. The pharmacokinetic parameters of the population model were as follows (geometric mean ± SD): metabolic clearance (CLm) 1.57 ± 3.71 L/h, volume of distribution (Vd)0.30 ± 0.05 $L*kg^{-1}$ lean body mass, drug clearance – creatinine clearance ratio (fr) 0.02 ± 0.13, gamma distribution rate constant (Ktr_po) 2.18 ± 1.14, gamma distribution shape factor (n_po) 2.15 ± 0.39. Free fraction of sulfamethoxazole was 0.3, but ranged between 0.2-0.4. The median value of the MICs was 9.5 mg/L (IQR, 4.75-9.5) and of *ƒ* AUC/MIC ratio was 14.3 (IQR, 13.0-17.5). The percentage of *ƒ* T>MIC ranged between 43 and 100 % of the dosing interval. The PK and PD data from this study are useful to explore a future dosing regimen of co-trimoxazole for MDR-TB treatment.

INTRODUCTION

Tuberculosis (TB) annually still accounts for millions of cases of active disease and a significant number of deaths worldwide. Among patients who were reported to have TB in 2013, there were 1.1 million new cases of TB among HIV-positive patients and 480 000 cases of multidrug-resistant (MDR)-TB [1]. The prevalence of MDR-TB has reached epidemic levels and is increasing in Africa, Asia, and Eastern Europe [2], while the majority is not treated according to the WHO recommendations.

Standard MDR-TB treatment includes first line drugs, for which the causative strain appeared susceptible, plus an aminoglycoside and a fluoroquinolone, with additionally drugs from group 4 and 5 to complete the regimen [3]. Unfortunately, the use of second line drugs including injectables such as aminoglycosides [4] is inconvenient in high prevalence areas; requiring parenteral administration [5]. In addition, there are other disadvantages of second line drugs compared to the two first line drugs isoniazid and rifampin such as their costs and toxicity.

Co-trimoxazole; an antimicrobial drug that has been on the market since the late 1960ties, is cheap and relatively safe, is not registered for treatment of TB, but it could be active against MDR-TB [6]. Co-trimoxazole, a combination of trimethoprim and sulfamethoxazole is widely used for the prophylaxis and treatment of a range of other infectious diseases [7]. Also, in TB patients co-infected with the human immunodeficiency virus (HIV), 41% reduction in mortality was reported among patients receiving 960 mg co-trimoxazole in a randomized controlled trial in South Africa[8]. Another study in Switzerland confirmed that co-trimoxazole decreased the risk for development of TB in HIV-TB co-infected patients that did not receive combined Anti-Retroviral therapy (cART) and to a lesser extent in cART treated patients [9]. The occurrence of side effects after receiving 960 mg was similar in a placebo- and co-trimoxazole group in HIV-TB patients [10,11].

Recently, *in vitro* studies and observational clinical data showed promising antimicrobial activity of sulfamethoxazole against *Mycobacterium tuberculosis* strains, revealing a MIC range 4.75-≤38mg/L and inactivity of trimethoprim against this bacteria [12-16].

The renal excretion of unchanged sulfamethoxazole is limited to about 20%. Sulfamethoxazole is also acetylated by N-acetyltransferase into sulfamethoxazole-N-acetyl, which increases its solubility. The renal excretion of sulfamethoxazole-N-acetyl is the major pathway of sulfamethoxazole removal [17,18].

The pharmacodynamic properties of SXT are not completely clarified but scarce literature favors the ratio of the area under the free concentration time curve (f AUC) from 0 to 24 h to MIC, and the duration of time a free drug concentration remains above the minimum inhibitory concentration (T>MIC) as potentially predictive PK-PD indices for determining

the efficacy of sulfamethoxazole [11,21].

In a previous retrospective study evaluating 8 MDR-TB patients receiving sulfamethoxazole in a dose of 400-800 mg once daily, the pharmacokinetic parameters showed little variability [15].

Based on a target ratio of $fAUC_{0-24}$/MIC of 25 derived from other bacterial infections [19], and the safety data from studies in HIV-TB patients [8,11,20], supplemented with earlier data on pharmacokinetics from MDR-TB patients [15] and MIC values [21], we postulated that a dose of 960 mg once daily may serve as a suitable starting point for dose selection for MDR-TB treatment.

To explore if the PK/PD target was met, a prospective open label study, evaluating co-trimoxazole 960 mg once daily in drug sensitive TB patients, was performed.

PATIENTS AND METHODS

Study design

This study was a prospective, open-label single-arm study and was performed at the TB-unit of the University Medical Center Groningen, location Beatrixoord in Haren, The Netherlands. It was estimated that a sample size of 12 patients was sufficient to explore PK/PD target attainment after administration of SXT 960mg once daily. The study was approved by the medical ethical committee (METc 2013/195) and registered at clinical trials.gov (NCT01832987). The patients in this study received co-trimoxazole on top of their standard TB treatment (rifampicin, isoniazide, pyrazinamide, ethambutol) in a dose 960 mg orally for four to six days (in order to prevent blood sampling within the weekends) to reach steady state, since the half time is approximately 10 hours [22].

Patients

Subjects eligible for inclusion were culture confirmed TB patients aged 18 years and older. Patients were enrolled in this study after they provided written informed consent. The patients were excluded if they had shown hypersensitivity to sulfonamides or trimethoprim, were pregnant or providing breast -feeding, had preexisting renal dysfunction (serum creatinine clearance ≤15 ml/min) or gastrointestinal complaints like diarrhea and vomiting. Patients receiving angiotensin converting enzyme inhibitors, potassium–sparing diuretics, methotrexate, dofetilide, phenytoin, sulfonylureas (glibenclamide, gliclazide, glimepiride and tolbutamide) or procainamide hydrochloride were also excluded from study participation. TB patients, concomitantly receiving treatment with vitamin K antagonist (acenocoumarol)

were also excluded from participation in this study.

Additionally, the patients that had experienced an adverse effect to co-trimoxazole or similar antimicrobial drugs, patients with HIV or AIDS, severe damage to liver parenchyma, characterized by elevation of alanine-amino-transferase (ALAT; normal value < 45 U/l) and/or aspartate-amino-transferase (ASAT; normal value <40 U/l) three times the normal values or hematological disorders mainly anemia (hemoglobin level < 5.5 mmol/L), thrombocytopenia (leukocyte count >6 × 10^9/L) and agranulocytosis (granulocyte count <210*9/L) were also excluded.

Study procedures

Evaluation of medical chart of TB patients including demographic characteristics, underlying disease, localization of TB was done at day one of the study (baseline).

Co-trimoxazole 960 mg (Sandoz®; Salutas Pharma GmbH, Barleben, Germany) was given orally in a single daily dose after a light breakfast. Blood samples were collected before administration and at 1h, 2h, 3h, 4h, 5h, 6h, 8h and 24 hrs after co-trimoxazole administration after at least 4 days of treatment (I.e. at steady state) [22,23].

The concentrations of sulfamethoxazole in human plasma samples were analyzed by a validated liquid chromatography-tandem mass spectrometry (LS-MS/MS) as described in detail earlier [15]. Measurement of sodium, potassium and creatinine levels and platelet count of the participants were done at baseline.

Drug susceptibility testing (DST) was performed at the National Mycobacterial Reference Laboratory (National Institute for Public Health and the Environment [RIVM], Bilthoven the Netherlands) by the Middlebrook 7H10 agar dilution method [24]. As recommended by the European Committee on Antimicrobial Susceptibility Testing guidelines, the MIC of co-trimoxazole is expressed as trimethoprim: sulfamethoxazole in the ratio 1:19.

The unbound concentration of sulfamethoxazole in plasma ultra-filtrate was measured in the sample at 3 time points (2h, 4h and 24 hours). Individual pharmacokinetic parameters were calculated using MW\Pharm 3.81 KinFit module with a one-compartment model with lag time and a fixed estimated bioavailability of 1.

Population pharmacokinetics and model validation

The pharmacokinetic parameters were calculated using one compartmental analysis (MW\Pharm 3.81; Mediware, Groningen, The Netherlands), utilizing an iterative two-stage

Bayesian approach [25]. Based on these calculated parameters; a one compartmental population model was developed based on 800 mg sulfamethoxazole. The creatinine clearance was estimated using the Cockcroft-Gold formula [26]. The volume of distribution was normalized to the lean body mass (LBM). This model was optimized to fit the curves and to minimize the calculated AIC value.

A two-compartment model showed no significant improvement in fitting the sulfamethoxazole concentration over time curves. The clearance was calculated with CL = CL_m (metabolic clearance (L/h)) * BSA (body surface area (m^2)) /1.85 + fr (drug clearance – creatinine clearance ratio) * CLcr (creatinine clearance (L/h)).

Other descriptors in the formulas, such as the body weight, lean body mass and free fat mass did not improve the model fit based on the calculated Akaike information criterion (AIC) value. Also, the use of an allometric component b (standardized on 0.75) did not improve the model fit. The model was build using transit absorption rate with initial gamma distribution rate constant (ktr_po) and the gamma distribution shape factor (n_po) values of 2 ± 0.5 [27,28]. The bioavailability (F) was fixed to 1. The parameters of population model were assumed to be log-normally distributed and the variability in the pharmacokinetic parameters was calculated using Bootstrap analysis (n = 1000). The assay error was assumed to be normally distributed and was estimated at 0.1 + 0.1*C. Co-variate analysis was performed by MW\Pharm, assessing the influence of the age, weight, height, BSA, lean body mass and CLcr with the CLm, fr, V_d and K_a.

This population pharmacokinetic model was cross-validated using the n-1 method, where a model with one omitted patient was repeatedly used to calculate the $AUC_{0\text{-}24h}$ of this one omitted patient [29].

Furthermore, the model was externally validated using the PK data of a cohort of MDR-TB patients (n=8) using SXT 480 – 960 mg once daily (sulfamethoxazole dose: 400 – 800 mg) [15] .

Unbound concentrations of sulfamethoxazole were measured and were divided by the total concentration to find the free fraction of sulfamethoxazole, which was assumed to be comparable regardless of the concentration. Consequently, the unbound $AUC_{0\text{-}24h}$ was calculated by multiplying the total $AUC_{0\text{-}24h}$ by the average free fraction. In turn, free $AUC_{0\text{-}24h}$/MIC ratio was also determined based on the individual observations of the $AUC_{0\text{-}24h}$ multiplied by the average free fraction measured in 3 different samples of that particular patient. The time period in which the free concentration exceeded the MIC (T>MIC) of sulfamethoxazole was calculated. The maximum plasma concentration (C_{max}) and minimum plasma concentration (C_{min}) were assessed directly from the plasma concentration data.

Statistical analysis

The difference of the population pharmacokinetic data and the observed data were tested by calculating the Root Mean Square Error (RMSE) and by constructing a Bland-Altman plot. Furthermore, the n-1 model was also compared with the observed data using both techniques. Predictive value of this model for the earlier population was evaluated using the RMSE. Pharmacokinetic parameters were compared using Wilcoxon Signed Rank Tests using SPSS 20 (SPSS, Virginia, IL).

RESULTS

Patient characteristics

A total of 12 patients (10 males and 2 females) with a median age of 30 (Inter Quartile Range (IQR), 25-50) years were enrolled in this study. They had a median body mass index of 20.2 kg/m^2 (IQR, 18.7-21.9 kg/m^2). All patients received 960 mg of co-trimoxazole once daily, which was equal to a median dose of 13 (IQR 11.8- 14.2) mg/kg. Diagnosis of TB was confirmed by culture and/or molecular tests, ten patients were diagnosed with pulmonary TB and one patient was diagnosed with spinal (extra pulmonary) TB and one with both pulmonary and pleural and spinal (extra pulmonary) TB. Baseline characteristics of TB patients are shown in table 1.

TABLE 1- Baseline characteristics of TB patients receiving SXT (n=12)

Parameter	**Value (median± IQR)**
Age (yr)	30 (25-50) yr
Gender (Male/female)	10/2
Body mass index (kg/m²)	20.2 (18.7-22)
Ethnicity Europe Africa America ' middle east western pacific region	 6 3 1 1 1
Co- morbidity Smoking Alcohol abuse Illicit drug Diabetes mellitus Anemia Anorexia	 6 4 1 1 1 1
Localization of TB Pulmonary Extra pulmonary TB (pleural and spinal TB)	 11* 1
Other anti-TB drugs	Isoniazid, rifampicin, pyrazinamide, ethambutol, moxifloxacin
Sodium level (mmol/L)	140 (139-141.2)
Potassium level (mmol/L)	4 (3.7-4.2)
Creatinine clearance (ml/min/1.73 m2)	103.2 (106.0 – 112.0)
Platelet count (×10*⁹/L)	292 (235.5-394.7)

* One of 12 patients was diagnosed with both pulmonary- and extra pulmonary TB.

Observed kinetic parameters

The observed pharmacokinetic parameters, as calculated using a one-compartment model with lag-time and a fixed bioavailability of 1, are shown in table 2. A large interindividual deviation in the rate and onset of the absorption is observed, which explains the large absorption coefficient (K_a) variation. The elimination phase (k_{el}), however, was consistent and showed only little variation (median 0.09, IQR; 0.08 – 0.14 h^{-1}). The median distribution volume per lean body mass varied was calculated at 0.025 (IQR; 0.020 – 0.028) L/kg.

TABLE 2- Observed pharmacokinetic parameters of sulfamethoxazole (800 mg)

Parameter	Median (interquartile range)
AUC(mg/L*h) *1	566.6 (360.8 – 658.1)
CL (L/h)	1.34 (1.19 – 2.04)
V_d (L)	14.53 (11.82 – 16.82)
V_d/BW (L/kg)	0.23 (0.19 – 0.30)
T1/2 (h)	7.88 (4.95-8.27)
K_{el} (h^{-1})	0.09 (0.08 – 0.14)
K_a (h^{-1})	1.34 (0.72 – 4.49)
Lag time (h)	0.46 (0.17 – 0.63)
F	1 (fixed)

CL: clearance, V_d: volume of distribution, V_d/BW: volume of distribution divided by the body weight, $T_{1/2}$: half time, k: elimination constant, K_a: absorption constant, F: bioavailability, *1 calculated using the trapezium rule

Pharmacokinetics, Model validation and pharmacodynamics

The pharmacokinetic parameters of this population model are displayed in table 3.

TABLE 3- Pharmacokinetic parameters of the population model (95% confidence intervals obtained by bootstrap analysis)

Parameter	Mean (95% CI)	± st. dev. (95% CI)	Shrinkage
CL_m (L/h/1.85m^2)	1.57 (1.01 – 2.04)	3.71 (0.26 – 3.46)	-0.8
V_d (L*kg^{-1} lean body mass)	0.30 (0.25 – 0.39)	0.05 (0.02 – 0.11)	0.20
f_r	0.02 (0.00 – 0.10)	0.13 (0.00 – 0.33)	0.08
Ktr_po (h^{-1})	2.26 (1.21 – 6.36)	1.05 (0.28 – 2.57)	-0.02
N_po	2.12 (1.00 – 5.84)	0.73 (0.17 – 3.44)	0.51

CL_m: clearance, V_d: volume of distribution, f_r: drug clearance - creatinine clearance ratio, Ktr_po: gamma distribution rate constant, N_po: gamma distribution shape factor.

Curves fitted to the population pharmacokinetic model resulted in a median $AUC_{0\text{-}24h}$ of 458.35 mg/L*h (IQR 380.65 – 553.9 mg/L*h). The fitted curve with the 5% and 95% percentiles and the observations are displayed in figure 1.

The observed and model calculated sulfamethoxazole concentrations are displayed in figure 2a and a Bland Altman plot of the concentrations is shown in figure 2b.

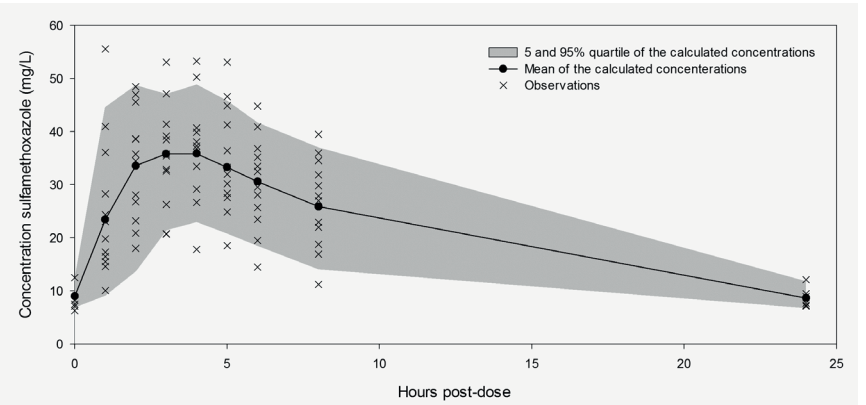

FIGURE 1- Sulfamethoxazole concentrations predicted by the model (line) and observations (crosses)

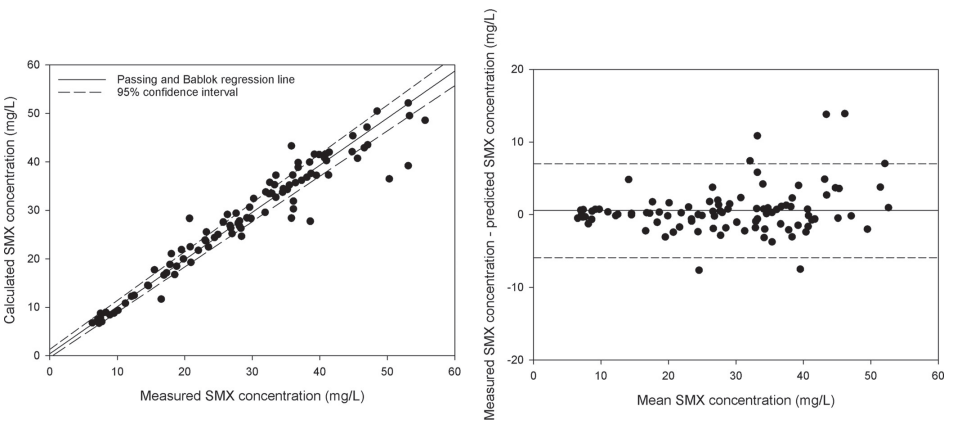

FIGURE 2 (**a)-** Passing and Bablok regression of the observed and model calculated Sulfamethoxazole concentrations (dashed lines, 95% confidence interval). **(b)** Bland-Altman plot of the measured concentrations versus the predicted concentration)/2 and measured concentration-predicted concentration

The mean value of free fraction of sulfamethoxazole that is responsible for antimicrobial activity was 0.3 (range; 0.2-0.4).

Covariate analysis indicated that none of the tested parameters (CLm, fr, Vd, ktr_po and n_po) was significantly correlated with age, weight, height, gender, BSA, lean body mass and CLcr ($P > 0.05$).

Thereafter, the model was cross-validated using the n-1 validation procedure. The RMSE in the AUC was calculated at 7.6 h*mg/L with a coefficient of variation (CV) of the RMSE of 1.7%. Pharmacokinetic parameters resulting from the model and the n-1 validation were statistically equal ($P < 0.05$), except for the fr ($P = 0.038$). However, the difference between the fr from all individuals fitted to the model and fr resulting from the n-1 validation was relatively small (median fr 0.00 v.s. 0.17). A Bland-Altman plot assessing the difference in AUC0-24h between the model and cross validation calculations is displayed in figure 3.

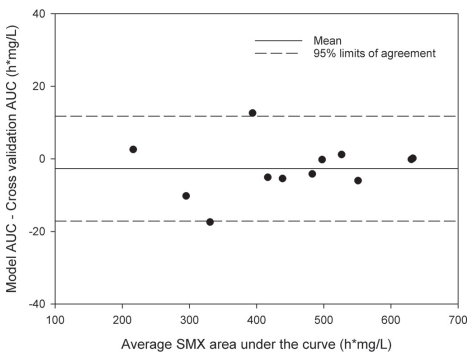

FIGURE 3-Bland-Altman plot cross-validation population pharmacokinetic model. Each dot represents a patient, where the coordinates of each point (x,y) are as follows:[modeled AUC+(n-1AUC)/2 and modeled AUC - (n-1AUC).

An additional model validation, in order to validate the model structure, was carried out by using the curves of eight patients as published earlier [15]. The clearance and volume of distribution were calculated based on these eight curves and compared to the pharmacokinetic parameters in the model of the earlier retrospective study [15]. The calculated values and their corresponding reported values are shown in table 4.

TABLE 4-Retrospective validation

Parameters	**Mean (± st. dev.)**		
	Model	**Alsaad et al. (15)**	**p***
CL (L/h)	1.18 ± 0.52	1.21 ± 0.43	0.674
V_d (L*kg^{-1} lean body mass)	0.30 ± 0.07	0.25 ± 0.04	0.050

CL: clearance, V_d: volume of distribution, K_a: absorption constant, F: bioavailability.

* Wilcoxon Signed Rank Test (two-tailed)

Drug susceptibility testing revealed that all of *M. tuberculosis* isolates from 11 patients were susceptible to sulfamethoxazole with median value of MICs of 9.5 (IQR, 4.75 - 9.5) mg/L. The median values of AUC/MIC and ƒAUC0-24h/MIC ratios after receiving 800 mg sulfamethoxazole were 51.2 h.mg/L (IQR, 35.7 - 66.6) and 14.3 h.mg/L (IQR, 13.0-17.5 h.mg/L) respectively. Thus, none of the patients had $ƒAUC_{0\text{-}24h}$/MIC ratio of sulfamethoxazole greater than 25. The percentage of free *T*>MIC ranged between 43%-100% of the dosing interval.

DISCUSSION

In our study, we investigated the PK-PD parameters of sulfamethoxazole in patients with drug-susceptible TB receiving SXT on top of first line drugs. This is relevant, as it can be considered to be one of the first steps in exploring SXT as a potential alternative drug in the treatment for MDR-TB [6,15].

The observed pharmacokinetic parameters are calculated using a one-compartment model with lag time and a fixed bioavailability of 1 [19,15]. The absorption constant K_a showed to be variable with a large deviation, which might indicate that the absorption could be influenced by food intake. For this reason, we added a Bayesian simulated lag time to the population pharmacokinetic model to reduce K_a variability. Nevertheless, there was a high variability in the observed pharmacokinetic absorption constant. Therefore, we can conclude that the time to the maximum blood concentration is not homogeneous in our population.

The model was also validated by refitting new population model using the curves collected during a retrospective study. The difference in CL and Vd of both models was statistically not significant (P= or > 0.05, table 4). However, the K_a found in our report was higher than the retrospective study of Alsaad et al [15]. This can be explained by the fact that a one-compartment model without lag time was used, which may have caused the difference in the absorption constant.

Our results show that the median values of the exposure (AUC0-24) in drug-susceptible TB patients are lower than earlier reported data obtained from eight MDR-TB patients [15]. The lower $AUC_{0\text{-}24}$ might be explained by drug-drug interaction with rifampicin, as this drug reduced the $AUC_{0\text{-}24}$ of sulfamethoxazole with 23% in an earlier study [30,31] when co-administered in HIV patients. Interestingly, this percentage is comparable to the difference (22.2 %) in AUC between drug susceptible TB patients in this study and MDR-TB patients from our retrospective study [15].

Moreover, the free (non-bound) fraction of sulfamethoxazole is responsible for the

antimicrobial activity [32]. The unbound fraction of sulfamethoxazole in TB patients was comparable to that in healthy human subjects in an earlier study [32]. The similarity in free fraction between TB patients and healthy subjects can therefore not explain the difference in AUC between healthy subjects and our patients.

In our study we measured the concentrations of the drug only in serum rather than in epithelial lining fluid. For pulmonary infections, the concentration of drug in epithelial lining fluid (ELF) and alveolar macrophage cells may represent the antibiotic activity in the infection site. Unfortunately, sampling by bronchoscopic broncho-alveolar lavage without a clinical indication to collect ELF was not feasible. Therefore, the free concentration of drug in serum is the most reliable for the time being, as it is correlated with patient outcome [33].

The MIC values of sulfamethoxazole against clinical isolates of *M. tuberculosis* were similar to the MICs in previous studies [12-16]. We have previously shown that the MIC of sulfamethoxazole is not significantly different in MDR-TB patients versus drug susceptible TB patients [16]. The MICs reported in this study are therefore representative for the sensitivity of multidrug resistant *M. tuberculosis*.

The percentage of f *T*>MIC in our study is more than 43% of the dosing interval. This is comparable with other drugs with anti-TB activity, like pyrazinamide in TB patients [34]. Because of lack of data on the clinically validated values of f AUC/MIC and percentage of free T>MIC in TB patients, these parameters in our patients could be used as a starting point to evaluate the efficacy of sulfamethoxazole.

This study has several limitations. The PK parameters of sulfamethoxazole were determined while sulfamethoxazole was administered in combination with rifampicin, which likely resulted in a 23% lower exposure[23,24]. Another limitation is that absolute bioavailability could not be calculated as the sulfamethoxazole exposure after oral administration was not compared with the administration of an intravenous dose. Additionally, the study was not designed to assess efficacy/outcome of sulfamethoxazole and therefore the PK-PD target index could not be evaluated in this study.

One of the possibilities to get more knowledge about the PK-PD index including f AUC/MIC and T>MIC in relation to efficacy is to test sulfamethoxazole in a hollow-fiber infection model. Based on that target, the dose selection for an explorative phase II study in MDR-TB patients can be performed.

In summary, this is the first report evaluating the PK-PD parameters of sulfamethoxazole in drug-susceptible TB patients. The established PK, encouraging antimicrobial activity of sulfamethoxazole against *Mycobacterium tuberculosis* strains *in vitro* and safety profiles in humans make this drug a suitable therapeutic option for treatment of MDR-TB in the near future.

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Conflict of interest: none

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CHAPTER 6

DRIED BLOOD SPOT ANALYSIS FOR THERAPEUTIC DRUG MONITORING OF CO-TRIMOXAZOLE IN PATIENTS WITH TUBERCULOSIS

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ABSTRACT

Co-trimoxazole, consisting of sulfamethoxazole and trimethoprim, is used for the prevention of infections with *Pneumocystis jiroveci* pneumonia (PCP) in HIV-infected patients. In addition, data from HIV-TB co infected patients suggest that co-trimoxazole may be of added value in the treatment of TB.

Unfortunately, the blood concentrations of sulfamethoxazole are highly variable between individuals, and this variation may attribute to its toxicity. Dried blood spots (DBS) have shown to be a reliable alternative to venous blood sampling, notably due to simple collection strategy and superior sample stability.

We developed a liquid chromatography tandem mass spectrometry analysis of sulfamethoxazole and its toxic metabolite, sulfamethoxazole-N-acetyl to quantify both compounds in DBS cards. The method was validated according to FDA and EMA guidelines and clinically validated.

The stability of the compounds on the dried blood spots was acceptable with a bias of maximal -7.5% and -7.0% after 14 days at 50 °C and 1 month at 37 °C, respectively. The median difference in the area under the curve calculated on DBS compared to plasma samples was -5.8% (IQR -6.25 - -0.13%).

This newly developed method showed to be reliable and robust and was fully validated. The stability of both compounds was sufficient to transport DBS cards from developing countries to a sophisticated laboratory to perform TDM. This method can be used in daily patient care and in future prospective pharmacokinetic studies exploring the use of sulfamethoxazole for TB treatment.

INTRODUCTION

Tuberculosis (TB) is a life threatening disease killing approximately 1.5 million people every year [1, 2]. TB is an infectious disease caused by *Mycobacterium tuberculosis*. Multidrug resistant (MDR) *M. tuberculosis* is resistant to at least isoniazid and rifampicin, the two most powerful first line anti-tuberculosis drugs [3]. The spread of MDR-TB is increasing, with an estimated 310,000 new infections in 2011 [1]. Therefore, much effort is needed to find new treatment options.

A potential candidate for MDR-TB is co-trimoxazole; a classic drug used against many types of infections [4-7]. Co-trimoxazole consists of trimethoprim and sulfamethoxazole. Trimethoprim is not active against *M. tuberculosis* but sulfamethoxazole showed *in vitro* activity against both TB and MDR-TB [8, 9]. A recent prospective cohort study showed that co-trimoxazole prophylaxis against *Pneumocystis jiroveci* pneumonia (PCP) in the treatment of HIV reduced the incidence of TB [10]. These considerations make this drug a potential candidate for the treatment of TB [4]. Co-trimoxazole has been shown to be effective in the prophylaxis of PCP in HIV-infected patients with a CD_4 count below 200 / mm^3 [11]. It is therefore implemented in HIV treatment guidelines as standard care until there is evidence of immune recovery [11]. Although a fixed dose has been recommended, serum concentrations vary considerably between individuals. In patients with chronic liver disease, increased serum concentrations of sulfamethoxazole were found, correlating with a larger likelihood of toxicity [12]. Furthermore, sulfamethoxazole is metabolized by N-acetyltransferase to Sulfamethoxazole-N-acetyl, which has the tendency to precipitate due to concentration in the kidneys. This has a detrimental effect on the renal function [13]. A general advice is to drink 2 litres of water per day to avoid this.

To ensure that therapy with sulfamethoxazole is effective, the exposure to the drug needs to be high enough [14]. This exposure can be quantified using the area under the pharmacokinetic (PK) curve for 24 hours ($AUC_{0\text{-}24h}$). The plasma $AUC_{0\text{-}24h}$ is a surrogate marker for the concentration at the site of the infection and the exposure to the infecting micro-organisms. To have a surrogate marker for the efficacy, and toxicity, the $AUC_{0\text{-}24h}$ needs to be related to the minimal inhibitory concentration of the micro-organism (MIC, $AUC_{0\text{-}24h}$/MIC) [7, 14].

Dried blood spot (DBS) monitoring is a simple procedure to collect blood from a patient [15]. A DBS can be made with a few blood drops on a sheet of absorbent paper. The concentration of the drug of interest is subsequently measured in the dried blood spot. This method enables measuring blood levels of patients in outpatient clinics and in remote areas. The DBS can be easily stored and sent to a laboratory because of high sample stability [16]. From our own experience, it takes only a week to send a DBS card through

local post services from the rural country Ghana to our laboratory in The Netherlands with a 100% delivery rate up to now (n = 6, unpublished data). With the combination of high sample stability and easy sampling, therapeutic drug monitoring is feasible in rural parts of the world with a high frequency of HIV and/or TB.

Another additional advantage is reduced discomfort for patients. Moreover, medical personnel can take DBS samples after limited additional training. DBS analysis of anti-TB drugs is increasingly proposed to replace venous sampling for therapeutic drug monitoring (TDM) [17-21]. Several analytical DBS procedures for TB drugs have already been published [22-24].

Although DBS seems a simple procedure in the field, method validation is not and requires additional validation for the effect of the haematocrit value and blood spot volume [16]. A higher haematocrit value results in a higher blood viscosity and affects the flux and diffusion properties of the blood [15]. This may result in a positive analytical bias [15].

The objective of this study was to develop and validate a simple method to detect and quantify sulfamethoxazole and Sulfamethoxazole-N-acetyl simultaneously in dried blood spots suitable for TDM. The DBS procedure was also evaluated in a prospective study.

PATIENTS AND METHODS

Patients

TB patients between 18 and 64 years of age with culture confirmed drug-susceptible *M. tuberculosis* were eligible for this prospective clinical trial. Co-trimoxazole 960 mg daily was added for 4 to 6 consecutive days to the standard treatment of TB to obtain a steady state concentration situation. On the 5th (± 1) day, three dried blood spots were collected 1h, 5h and 8h post-dosage at the same time point as regular venous serum samples were taken. The study was approved by the local ethical committee (METC 2013/195) and registered at Clinicaltrials.gov (NCT01832987). Patients that participated in the study gave written informed consent**.**

Materials

Sulfamethoxazole was purchased from Sigma Aldrich (MO, USA). Sulfamethoxazole-N-acetyl was obtained from Santa Cruz (TX, USA). The deuterated internal standards sulfamethoxazole-D4 and sulfamethoxazole-N-acetyl-D4 were purchased from Alsachim (Illkirch Graffenstaden, France) and Santa Cruz (TX, USA), respectively.

Ultrapure water was produced with a Milli-Q system (Millipore Corporation, MA, USA).

Ammonium acetate and acetic acid were both obtained from Merck (NJ, USA), while trifluoroacetic acid and acetonitrile LC-MS were purchased from Biosolve (Dieuze, France). Methanol was retrieved from Merck (NJ, USA). Whatman DMPK type C cards (Whatman, Kent, UK) were used for spotting the blood [25].

LC-MS/MS

The LC-MS/MS apparatus and conditions were identical to the serum and plasma analysis, as published earlier [26]. In short, liquid chromatography separation took place with a Scientific Hypurity Aquastar C18 (50*2.1 mm, 5µm particles) column in a system with a Finnigan Surveyor MS Pump Plus and Finnigan Surveyor Autosampler Plus. Gradient elution with three mobile phases was applied to reduce the run-time to three minutes with a flow rate of 500 µL/min [26].

A TSQ Quantum Access Max (TSQ Quantum, Thermo Scientific, San Jose, CA, USA) tandem quadrupole mass spectrometer was used for both the serum and DBS MS/MS analysis. The spray voltage was 3500V, sheath and auxiliary gas pressure was set to 35 and 10 bar and the capillary temperature was set to 350 °C.

To perform the analysis, the most sensitive ion transitions were used: 254.0 → 156.1 (sulfamethoxazole), 258.1 → 160.1 (sulfamethoxazole-D4), 296.1 → 198.0 (Sulfamethoxazole-N-acetyl), 300.1 → 202.1 (Sulfamethoxazole-N-acetyl-D4).[26]

Preparation of solutions and QCs

The internal standard solution was composed of 0.2 mg/L sulfamethoxazole-D4 and 2.0 mg/L Sulfamethoxazole-N-acetyl-D4 in methanol/water (80/20). Quality control blood samples were prepared in blood consisted of LLOQ (2 mg/L), LOW (10 mg/L), MED (40 mg/L) and HIGH (80 mg/L) samples of sulfamethoxazole and Sulfamethoxazole-N-acetyl. DBS QC samples were produced by pipetting 50 µL of the quality control blood samples on a DBS card and drying it for three hours.

Sample preparation

A venous dried blood spot (VDBS) was made by pipetting 50 µL of the venous plain blood on a DBS paper. To analyse both VDBS and DBS, a paper disc of 8 mm was punched out each blood spot on the DBS. The IS solution was added (250 µL) and the sample was vortexed. After vortexing, the sample was placed in an ultrasonic bath for 10 minutes. Afterwards, the sample was vortexed again for one minute and subsequently centrifuged

for 5 minutes at 10,000 rpm. Of the extract, 200 µL was transferred in another vial, 500 µL ultra-pure water was added and 10 µL was subsequently injected on the column.

Method validation

Each calibration line consisted of eight different concentrations, as shown in table 1.

TABLE 1- Calibration lines

Compound	Slope (± st. dev)	Intercept (± st. dev)	Corr. coefficient	Regr. coefficient
Sulfamethoxazole	0.392 (± 0.004)	-0.009 (± 0.023)	0.999	0.997
Sulfamethoxazole-N-acetyl	0.112 (± 0.002)	0.005 (± 0.008)	0.998	0.996

Each concentration was prepared and measured in triplicate. Both sulfamethoxazole and sulfamethoxazole-N-acetyl were measured in the range of 2.0 – 100.0 mg/L. Calibration curves were corrected for the internal standard based on peak area. The slope, intercept and correlation and regression coefficient were calculated by ANOVA.

Validation was performed according to the FDA and EMA guidelines. Selectivity was determined by analysing six blank blood spots retrieved from volunteers on our lab. All samples were tested for peaks on the retention times of sulfamethoxazole and Sulfamethoxazole-N-acetyl. Ion suppression was tested using a constant infusion test.

Accuracy (coefficient of variation (CV)) was investigated by the analysis of LLOQ (2.0 mg/L), LOW (10.0 mg/L), MED (40.0 mg/L) and HIGH (80.0 mg/L) concentrations of both analytes in fivefold on three consecutive days. Precision (bias (%)) was examined within-run and between-run with the data from the accuracy determination. Dilution integrity was also tested during these three consecutive days by spiking a DBS card with 200 mg/L in fivefold with sulfamethoxazole or sulfamethoxazole-N-acetyl. The extract was diluted 1:10 and analysed. The within-day and between-day precision were calculated using ANOVA.

The matrix effect was determined by spiking extracts of blank DBS with LOW, MED and HIGH QC concentrations of sulfamethoxazole or sulfamethoxazole-N-acetyl and comparing them with the peak area of spiked extraction fluid. The recovery was calculated by dividing the peak area of LOW, MED and HIGH concentration spiked DBS by the peak area of spiked extract of a blank DBS. Process efficacy was determined by dividing the peak area of a DBS spiked with LOW, MED and HIGH QC concentration divided by the peak

area of spiked extraction fluid.

The stability assessment was performed by analysing spiked dried blood spots (LOW and HIGH QC concentrations) stored for 14 days at 20, 37 and 50 °C and one month at 20 and 37 °C. The peak area of analysed dried blood spots was compared with the peak area of freshly prepared samples.

Influence of haematocrit and blood spot volume

The influence of the haematocrit (Hct) value on the accuracy and precision was additionally assessed. In tuberculosis patients, an Hct value of 35% was expected [27]. We tested therefore Hct values of 20, 25, 30, 35, 40, 45 and 50%. The influence of the volume of the blood spot was also assessed by varying the blood spot volumes from 30, 50, 70 to 90 µl.

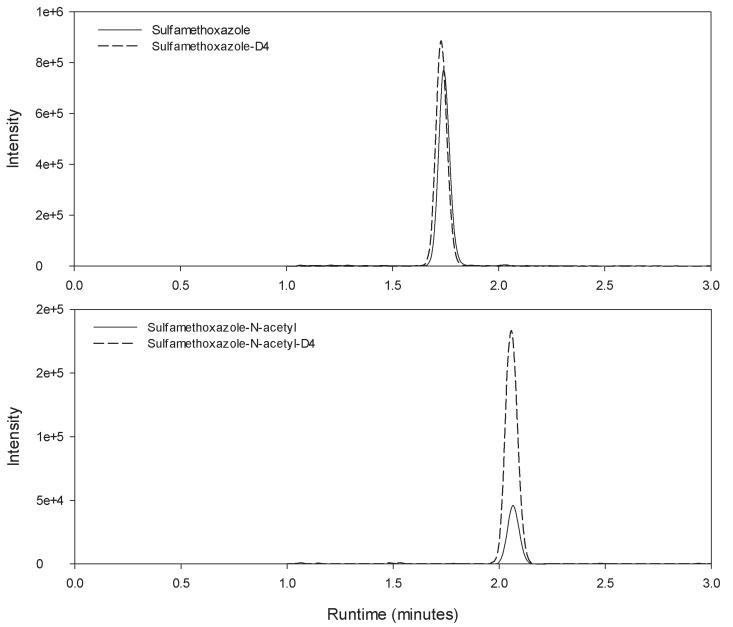
Clinical validation

Venous blood, VDBS and DBS concentrations were compared with a Bland-Altman plot and Passing and Bablok regression. According to EMA guidelines, the difference between DBS and serum concentrations should be within 20% in 67% of the cases [28]. Plots were constructed with SigmaPlot version 12 (Systat Software Inc., San Jose, CA, USA). Passing and Bablok regression was calculated using Medcalc version 15.8 (MedCalc Software bvba, Ostend, Belgium). The area under the curve (AUC_{0-24h}) of sulfamethoxazole was calculated based on the measured DBS concentrations using a validated one-compartment population pharmacokinetic model. The AUC_{0-24h} based on the DBS samples was compared with the AUC_{0-24h} calculated with full curves.

RESULTS

Method validation

No peaks were observed in six lots of blank samples at the retention times of the analytes (as shown in figure 1 (supplemental data)). No ion suppression was observed. The details of all three calibration lines are displayed in table 1. The correlation and regression coefficients were all >0.99.



SUPPLEMENTARY FIGURE 1- Chromatogram of sulfamethoxazole (LLOQ), sulfamethoxazole-D4, sulfamethoxazole-N-acetyl(LLOQ) and sulfamethoxazole-N-acetyl-D4.

The accuracy in measuring sulfamethoxazole and sulfamethoxazole-N-acetyl varied between -14.2 – 2.4% (as shown in table 2). The coefficient of variation between the measurements was 2.9 – 6.9% within-day and 0.0 – 9.5% between three days. The mean bias in determining the dilution integrity of sulfamethoxazole and sulfamethoxazole-N-acetyl was 1% and -4% with an overall CV of 9.5% and 6.6%, respectively. Both accuracy and precision fell within the FDA and EMA defined limits of 15% (LLOQ: 20%).

TABLE 2- Validation results

Criteria	Concentration level			
	LLOQ	**LOW**	**MED**	**HIGH**
Nominal concentration (mg/L)				
Sulfamethoxazole	2.0	10.0	40.0	80.0
Sulfamethoxazole-N-acetyl	2.0	10.0	40.0	80.0
Accuracy (bias (%))				
Sulfamethoxazole	-6.9	-2.4	0.9	2.4
Sulfamethoxazole-N-acetyl	-14.2	-3.0	0.5	0.2
Within-day precision (CV (%))				
Sulfamethoxazole	3.3	2.9	3.5	3.6
Sulfamethoxazole-N-acetyl	6.9	6.9	5.8	6.0
Between-day precision (CV (%))				
Sulfamethoxazole	9.5	4.5	3.7	2.9
Sulfamethoxazole-N-acetyl	0.0	3.0	3.7	1.0
Matrix effect (bias (%))				
Sulfamethoxazole	n.d.	0.8	2.1	5.1
Sulfamethoxazole-N-acetyl	n.d.	0.1	3.1	6.7
Recovery (bias (%))				
Sulfamethoxazole	n.d.	86.4	96.1	87.5
Sulfamethoxazole-N-acetyl	n.d.	93.2	103.7	89.6
Autosampler stability (7 days) (bias %)				
Sulfamethoxazole	n.d.	-1.6	n.d.	-2.8
Sulfamethoxazole-N-acetyl	n.d.	-3.0	n.d.	-5.4

The results of the matrix effects and recovery evaluation are shown in table 1. The limits of the FDA and EMA were met at all three concentration levels. The recovery and matrix effects varied from 86.4 – 103.7% and 0.1 – 6.7%, respectively, and complied with the FDA and EMA defined limits.

The autosampler stability of sulfamethoxazole and sulfamethoxazole-N-acetyl was determined after 7 days. The bias was -1.6 – -5.4% compared to the nominal concentration.

The stability of the two analytes on the DBS is evaluated at various storage conditions during 14 days and one month as shown in figure 2. After 1 month at 37°C, the bias calculated for sulfamethoxazole and sulfamethoxazole-N-acetyl -7.0 – 3.9%. Storage at 50°C for 14 days resulted in a deviation of -7.5 – -2.9% in comparison with the nominal concentration.

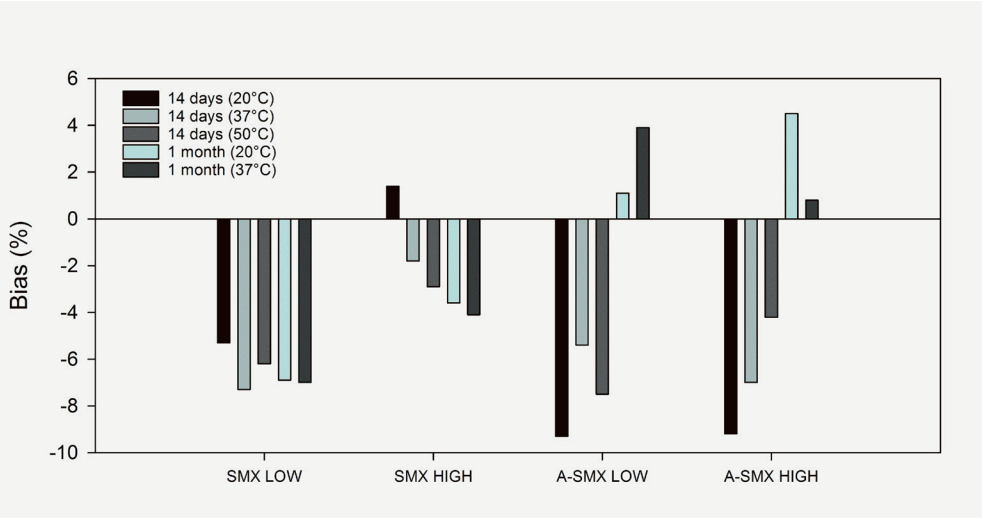


FIGURE 2-Stability of Sulfamethoxazole (SMX) and Sulfamethoxazole -N-acetyl(A-SMX) at LOW and HIGH QC concentration exposed to various storage conditions.

Influence of haematocrit and blood spot volume

The influence of the haematocrit (hct) value is displayed in figure 1. At a hct value of 20%, bias varied from -15.3 – -8.8% for all analytes. A bias of 4.0 – 5.1% was found at a relatively high haematocrit value of 50%. The bias caused by varying the blood spot volume was -3.2 – 6.1%, as shown in figure 3.

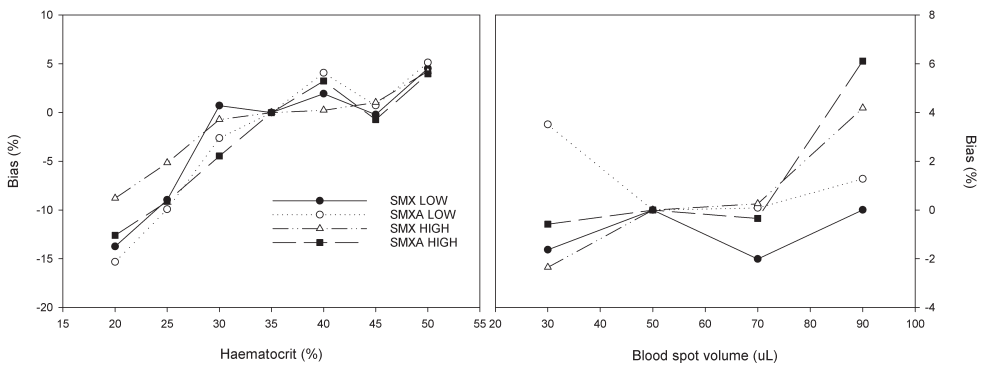


FIGURE 3-Influence of the haematocrit and blood spot volume on the measured concentration. SMX: sulfamethoxazole, SMXA: sulfamethoxazole-N-acetyl.

Clinical validation

In total 12 patients were prospectively enrolled in this study. The median age was 31 (IQR: 26 - 52) years with a median height of 175 cm (IQR; 168.2 – 180.0) and a median body weight of 61.5 kg (IQR; 56.4 – 67.2). All received 960 mg co-trimoxazole once daily.

Overall, 21 DBS samples of 9 patients were used to validate the method of analysis. The median haematocrit-corrected DBS concentrations were 20.8, 25.4 and 19.7 mg/L at 1, 5 and 8 hours' post-dose. Median serum concentrations were 18.5, 35.0 and 30.8 mg/L, respectively. The correlation between serum and haematocrit-corrected DBS concentrations was best described by: serum = -5.36 + 1.57 x DBS (Passing and Bablok regression). The 95% confidence interval of the intercept and slope was -11.43 – 0.40 and 1.31 – 1.85, respectively. All haematocrit-corrected DBS, VDBS and serum sulfamethoxazole concentrations are displayed in figure 4a.

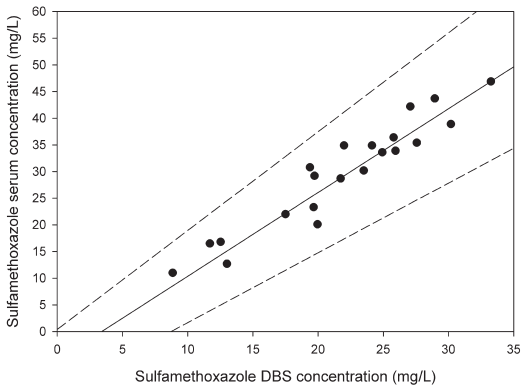


Figure 4a-Passing and Bablok correlation between sulfamethoxazole serum concentration and the haematocrit corrected DBS concentration (95% CI: dotted lines). Intercept: -5.36 (95% CI: -11.43 – 0.40), slope:1.57 (95% CI: 1.31 – 1.85)

After correction using the proposed regression formula, three of all 21 DBS concentrations deviated more than 20% with the serum concentration (77.7%, 79.0% and 129.4%). In addition, we calculated the regression formula with only the samples 5 and 8 hours post-dose. This relationship was best described by: serum = -2.88 (95% CI: -11.02 – 2.97) + 1.57 (95% CI: 1.30 – 1.89) x DBS. None of the back calculated serum concentrations at 5 and 8 hours' post-dose differed more than 20% with the actual serum concentration using this regression formula.

For sulfamethoxazole-N-acetyl, the median haematocrit-corrected DBS concentrations were 3.3, 4.7 and 5.9 mg/L at 1, 5 and 8 hours post-dose. Median serum concentrations were quantified at 6.0, 10.0 and 9.9 mg/L. The correlation between the haematocrit-corrected

DBS concentration and serum concentration was best described by: serum concentration (mg/L) = 0.50 (95% CI -6.99 - 2.59) + 1.77 (95% CI 1.26 – 3.15) x DBS concentration (mg/L), (Passing and Bablok regression) as shown in figure 4b. The difference between the corrected DBS and the serum concentration was >20% in 6 of 17 cases (71.1% – 154.5%).

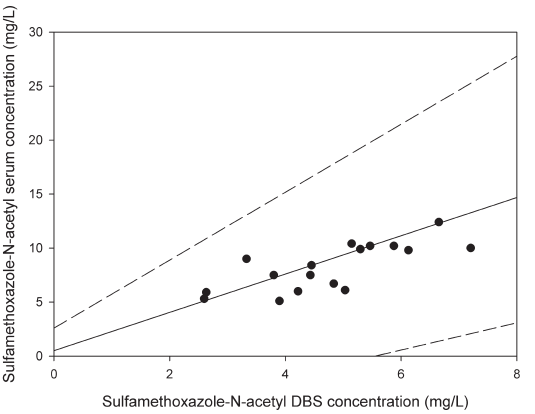


FIGURE 4B- Correlation between sulfamethoxazole-N-acetyl serum concentration and the haematocrit corrected DBS concentration (95% CI: dotted lines). Intercept: 0.50 (95% CI -6.99 - 2.59), slope: 1.77 (95% CI 1.26 – 3.15)

The median difference in $AUC_{0\text{-}24h}$ calculated on the DBS samples was -5.8% (IQR -6.25 - -0.13%) in comparison with full serum curves. A Bland Altman plot is displayed in figure 4c. Two DBS-based $AUC_{0\text{-}24h}$ differed more than 20% (-23.7% - -22.8%). The coefficient of variation in the root mean squared error (CV(RMSE)) was 9.7%.

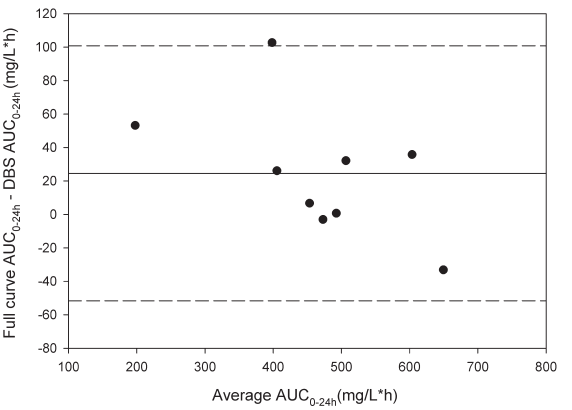


FIGURE 4C-Bland Altman plot of the AUC0-24h estimated based on full serum curves vs. based on DBS sampling.

DISCUSSION

We developed a method to analyse sulfamethoxazole and sulfamethoxazole-N-acetyl in dried blood spots which was successfully validated based on the FDA and EMA guidelines on bioanalytical validation [28, 29]. Clinical evaluation showed that estimating the AUC_{0-24h} with DBS samples provides comparable results with traditional venous blood sampling, indicating that this method is suitable for daily patient care.

Both the haematocrit and blood spot volume influence the quantification of analytes on DBS cards [24]. The haematocrit value was obtained from all patients and was used to correct the analytical result. Bias caused by varying blood spot volumes was within 6% and was considered negligible.

Due to compatibility with the serum and plasma analysis, we used identical mass transitions for all analytes of interest [26]. One problem which occurred during the development of the analytical method was the limited separation of sulfamethoxazole and sulfamethoxazole-N-acetyl. This problem was solved by adding 500 µL ultrapure water to the extract, increasing the hydrophilicity of the sample, which improved the separation. We used a gradient elution in order to further improve separation, and to minimize the total time of analysis to three minutes. In addition, we optimized the sample preparation turnover by comparing a 10 minute with a 60 minutes' ultrasonic bath. No difference in response was observed.

One of the major advantages of this dried blood spot technique is the simplified logistics since no precautions concerning storage environment are required. The bias in concentration after 14 days at 50 °C or 1 month at 37 °C did not exceed 15%, indicating that extreme temperatures do not affect the analytical result. This indicates that this method is also suitable in countries with an extreme climate without cold chain transport requirements. With this method, TDM is within reach in developing countries when the DBS samples are transported to a sophisticated laboratory for analysis and TDM.

The clinical validation of the analysis of sulfamethoxazole on DBS cards resulted in three deviations >20%, which meets the EMA requirements on cross-validation [28]. When using a regression formula based on the samples withdrawn 5 and 8 hours' post-dose, no deviations >20% are observed in the calculated serum concentration based on the DBS concentration versus the true serum concentration. This indicates that the peripheral distribution of sulfamethoxazole is not complete one hour after ingestion of the drug. When all DBS results were entered in our pharmacokinetic model, two deviations of -23% and -24% in the AUC_{0-24h} were observed compared to the AUC_{0-24h} calculated on full serum curves. Furthermore, the RMSE was calculated at 9.7%, indicating that predicting the AUC_{0-24h} of sulfamethoxazole with this DBS method is reliable and suitable for clinical use.

The sulfamethoxazole-N-acetyl concentration determination is only to detect toxicity leading to renal toxicity. This analysis seems less accurate than the analysis of sulfamethoxazole itself. The difference between DBS concentration and serum concentrations might be caused by a large inter- and intraindividual variation in peripheral sulfamethoxazole-N-acetyl concentration. However, the concentration of the DBS cards was higher than the serum concentrations in cases where the deviation was >20%. Therefore, the sulfamethoxazole-N-acetyl concentration may be over-estimated using this methodology that may result in an overestimation of possible toxic concentrations. When an increase of sulfamethoxazole-N-acetyl is observed, additional clinical evaluation and reanalysis of sulfamethoxazole-N-acetyl in venous blood is needed to evaluate renal toxicity. Although serum creatinine is also feasible using dried blood spots [30], it is unfortunately no measure of renal toxicity as co-trimoxazole influences tubular excretion of creatinine [31]. Therefore, measurement of an additional marker, such as Cystatin C, is required to confirm renal toxicity [32]. Cystatin C could also be quantified using DBS [33].

The method that we developed and validated is suitable for analysis of sulfamethoxazole and sulfamethoxazole-N-acetyl and is validated according the FDA and EMA guidelines on sensitivity, selectivity, linearity, accuracy, precision and stability. One-point sampling combined with this DBS method provides a convenient and reliable method to assess the individual pharmacokinetics of sulfamethoxazole in the treatment of various infectious diseases, such as PCP. In addition, this method can be used in further prospective trials to assess sulfamethoxazole exposure.

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Nothing to declare.

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CHAPTER 7

DISCUSSION & FUTURE PERSPECTIVES

Worldwide, the TB epidemic is in a crisis. TB control has largely failed to meet the Millennium Development Goals, and the newly drafted sustainable Development Goals for the next decades are unlikely to be met with current tools to fight TB [1, 2].

Current TB drug treatment regimens are challenged by increasing inhibitory concentrations for first– as well as second line TB drugs, with subsequently decreasing efficacy [3, 4].

Toxicity of most second-line drugs challenges doctors and patients to comply with treatment. Indeed, new TB treatment regimens are urgently needed. Only a few new TB products are in the pipeline and only two novel products have recently entered the market – bedaquiline and delamanid. For these new drugs, the long-term outcome has not been evaluated to date; early bactericidal activity is excellent but the largest challenge in TB treatment is to eradicate slowly replicating, metabolically inactive so-called 'persister' organisms. Rifampicin, in combination with pyrazinamide, provides the sterilizing capacity to allow for treatment duration of six months only, while fluoroquinolones e.g., moxifloxacin, have not been shown to further reduce treatment duration [5]. Discovery of new TB drugs is a difficult and expensive process and it takes a long time before new drugs reach the market.

Apart from novel drugs that have already been developed for other infections and have been shown to have efficacy for TB in vitro and in vivo should therefore be considered for uptake in existing TB. Moreover, such drugs should urgently be evaluated for their potential role in fighting multidrug resistance tuberculosis (MDR-TB).

Co-trimoxazole (SXT) is one such old drug that is already available in the market and that has not been listed in WHO guidelines but it clearly has anti-TB activity. This drug could play an important role in the treatment of MDR-TB patients as an additive drug in a combination therapy [6]. To better understand the desired activity of this drug against MDR-TB, more knowledge about the safety, efficacy and accurate quantification of SXT in biological fluid is required. Therefore, re-inventing of SXT using modern methods and strategy is needed [7]. This drug has desired characteristics including good oral bioavailability following oral administration, penetration into cerebro-spinal fluid, low incidence of side effects, good tolerability, an encouraging susceptibility profile of *M. tuberculosis,* minimal interactions with other anti-TB and human infected virus (HIV) drugs, few interactions with CYP450 enzymes; and low cost. Because of these characteristics, we were curious to evaluate the potential role of SXT in TB treatment.

Reviewing the literature on the pharmacokinetics (PK) and pharmacodynamics (PD) of SXT to determine the efficacy of SXT in the treatment of MDR-TB, we realized that only limited PK/PD data and information are available to date. Therefore, our aim was to evaluate these parameters in this thesis.

In our retrospective study the PK parameters of sulfamethoxazole *(*SMX), the effective part of SXT, were measured in MDR-TB patients. Plasma drug concentrations were lower compared to those measured in healthy volunteers and in patients infected with other diseases. This has been reported earlier for other drugs in TB patients who received anti-tuberculosis drugs [8]. We believe this is due to reduced bioavailability, probably as a result of low intestinal absorption in these patients and is less in line with an increased volume of distribution or increased drug elimination. Reduced bioavailability and subsequent reduced plasma drug concentrations over time increase the possibility of drug resistance without necessarily reducing adverse drug reactions. Reduced effectiveness of anti-TB drugs may ultimately increase the chance of treatment failure [9-11]. Another important consideration is the PK variability among TB patients that drives the $AUC_{0\text{-}24h}$/MIC ratio [12]. Indeed, this variability could be the main cause of the emergence of MDR-TB in the population, because this variation leads to low drug concentrations in several individuals; even if this results in effective treatment in most patients, it facilitates the emergence of drug resistance in others [13].

Fortunately, the population PK model of SMX from MDR-TB patients in our retrospective study showed a consistent PK profile and no significant variability in the area under the curve among these patients, the percentage of variation was 5.2% [14]. This contrasts with other drugs that have anti-TB activity like moxifloxacin, with a 9-fold inter-individual variability in AUC among MDR-TB patients [15].

Still there is a paucity of data about the susceptibility of *M. tuberculosis* strains from TB patients and a standardized guideline to measure the MIC of these bacteria against SMX is currently lacking as well; no break points have been determined. Also, the challenge of emergence of resistance to SMX when it is used in patients dually infected with HIV and TB who had received SXT for a long time as *Pneumocystis jiroveci pneumonia* (PCP) prophylaxis before TB was diagnosed has not been systematically studied. The results of SMX susceptibility testing of *M. tuberculosis* isolates between TB and TB-HIV and comparing with that of MDR-TB patient from the retrospective study were therefore promising, as no significant difference in MICs was observed among *M. tuberculosis* isolates in these patient groups.

SMX drug susceptibility of *M. tuberculosis* isolates from TB-HIV patients were tested. Although the result was beneficial, the risk of the development of active TB is a big challenge in these patients, and inadvertent mono-therapy with SMX for TB in patients in whom this diagnosis is missed presents a great risk to select drug-resistant mutants, thereby increasing the risk for the emergence of SMX-resistant clones of *M. tuberculosis*.

Besides, adverse events are common among individuals on anti-TB therapy, as are drug-drug interactions (DDIs) between antiretroviral and anti-TB drugs. For the effective

treatment of HIV-TB co-infection, pharmacokinetic DDIs should be avoided [16-18]. In comparison with new anti-TB drugs like Bedaquiline and DDIs with antiviral drugs like lopinavir and ritonavir [19], SMX has no clinically significant DDI when co-administered with indinavir sulfate [20, 21] and other antiviral drugs like didanosine [22] and zidovudine [23]. In low dosage as in PCP prophylaxis, SXT has mild and transient side effects in HIV-TB co-infected patients. All these benefits of our drug over the above-mentioned anti-TB drugs contribute toward the expected role of SXT in the treatment of TB in TB-HIV co-infected patients.

For most TB drugs, and probably also for SMX, the most important factor to estimate the required dosage of SMX is through calculating the optimal PK/PD parameters of SMX. Therefore, we developed a liquid chromatography tandem mass spectrometry (LC-MS). This method to determine the concentration of SMX and its metabolites in plasma and serum has multiple advantages. And we therefore used this method in a prospective study for specifying the minimally required, or optimal $AUC_{0\text{-}24}$/MIC ratio for SMX and testing our hypothesis that this ratio should exceed 25 to be effective in MDR-TB patients. In this study, we examined whether it is possible to achieve this target $AUC_{0\text{-}24}$/MIC ratio of 25. None of the patients with drug susceptible TB who received 960mg of SXT once daily had $fAUC_{0\text{-}24h}$/MIC ratio of SMX greater than 25. Therefore, we could not confirm our hypothesis that this ratio could exceed 25 after receiving 960 mg of SXT. However, the values $AUC_{0\text{-}24}$/MIC ratio and fT>MIC will be precisely confirmed in the near future. The free fraction of SMX was measured for the first time in TB patients and was comparable to that in healthy subjects and therefore this fraction does not explain the difference in AUC observed between these two groups.

SXT has been associated with side effects related to its high concentration in serum and insoluble Sulfamethoxazole-*N*-acetyl and trimethoprim [24]. In the absence of current dose recommendations and the use of this product in patients with limited treatment options, therapeutic drug monitoring (TDM) would be the suitable solution to all patients with active TB, to identify those with low drug exposure and to reduce the side effects of SMX metabolites to increase the therapeutic benefit and decrease toxicity. There is indeed a close relationship between the PK of drugs especially, low AUC and the emergence of acquired resistance to anti-TB drugs among HIV-TB co-infected patients [25]. Therefore, TDM is especially important in HIV-infected patients who receive antiretroviral and anti-TB therapy [26,13].

TDM has become easier to perform by using the Dried Blood Spot method that is to be preferred over classical blood sampling with its inherent difficulty in cold storage, and cold chain requirement until processing in a laboratory facility away from the point of care [27-30].

SXT was safe and well tolerated in MDR-TB patients compared to other drugs with anti-TB activity like linezolid. Though linezolid has been shown to be effective in the treatment of MDR-TB, serious side effects of linezolid and its cost could limit its use in the treatment, especially in low- and middle income countries that are typically highly endemic for TB and MDR-TB [31,32]. Based on the safety profile of SXT from our retrospective study in MDR-TB patients [14] and comparing it with safety of other drugs with anti-TB activity, SXT could be the safest alternative or adjunct anti-TB drug in TB treatment in the future.

Another benefit of SMX is the mechanism of action of sulfonamides including SMX as inhibitor of carbonic anhydrase (CA) enzymes including (mtCA 1, mtCA 2 and/or mtCA 3) which catalyzes the hydration of Carbon dioxide (CO2) and of dehydropteroate synthetase (DHPS) [33]. SMX is also inhibitor of dehydropteroate synthetase (DHPS), which catalyzes the addition of dihydropterindiphosphate to p-aminobenzoic acid (PABA). In experiments with overexpression of fol P1 and deletion of fol P2 genes in *M. tuberculosis,* these genes have been shown to play an important role in the anti-TB activity of SMX which is considered a clue for understanding the mechanism of action of SMX against *M. tuberculosis* [34].

In this thesis several aims were achieved. Limitations include the relatively small sample size, short duration of SXT administration to TB patients, gaps in our knowledge to assess the PK/PD target of SXT and lack of data about the role of SXT in the treatment of latent TB. Future clinical trials are urgently needed especially in low-and middle income countries where the TB burden is highest to confirm the bactericidal activity of SXT on actively replicating *M. tuberculosis*, to assess long-term safety and to explore the appropriate dose of SMX in MDR-TB patients. However, these trials for already existing drugs like SXT will be cheaper and easier to perform than those for the new anti-TB drugs, and clearly investigator-driven research could be designed to explore the possible role of SMX in MDR-TB. Development of novel drugs is a long process and few of these products reach the market. Furthermore, administration of SXT with other anti-TB drugs can affect the magnitude of $AUC_{0\text{-}24}$/MIC ratio and consequently prevent the assessment of the individual efficacy of SXT in TB treatment. Therefore, an *in vitro assay* (a hollow fiber infection model, *in vitro* macrophage systems) and *in vivo* models (mouse or rat model or guinea pig model) should be designed to determine PK/PD correlations of SXT alone and in combination with other anti-TB drugs to identify the optimal dose of SXT and in turn to provide the maximal bactericidal activity against *M. tuberculosis*. Our data on PK in TB patients is very useful for this study as actual exposure in TB patients can be simulated in the hollow fiber model instead of drug exposure derived from healthy volunteers. The activity of SMX on the inhibition of *M. tuberculosis* intra- and extracellularly as reported in a previous *in vitro* study showed that SMX was more effective against MDR strains of *M.*

tuberculosis while it showed less inhibition also in high doses inside the macrophages [35].

We focused only on the PK parameters associated to PD parameters of SXT against metabolically active, fast replicating *M. tuberculosis*, although the activity against dormant bacilli and persisters would be desirable. Dormancy can be defined as a state of low metabolic activity when the mycobacteria fail to form colonies. Consequently, the first step in the treatment of TB is to kill dormant or slowly replicating tubercle bacilli that can persist and start replicating if and when conditions favor reactivation [36,33]. Further insight into the potential application of SXT against latent TB has to be established in an aerobic *in vitro* test system such as the Wayne model.

SXT is widely used as prophylaxis in HIV patients who have a CD4 cell count <200 per mm^3, and stopped when CD4 count is consistently above 200 cells per mm^3 as a result of immune recovery in response to antiretroviral drugs [32]. Therefore, it is only ethical to evaluate the role of SXT in TB treatment in a prospective study in HIV-TB co-infected patients if CD4 count >200 cells per mm^3. Such study could evaluate the possible additional benefit of SXT group on top of HRZE.

We have concentrated mainly on TB in adult patients while special attention must be given to children, especially with HIV-TB co-infection. Although the occurrence of TB in children constitutes a highly variable proportion of the total TB burden in the population - between <5%-20% [37], the optimal treatment of TB in children has not been widely studied, while children are in fact much more susceptible to develop TB after infection, due to their less matured immune system. Administration of SXT to infected children prolongs the survival of these patients and reduces the incidence of respiratory infections and hospitalization [38,39]. HIV-infected children benefit more from the antibacterial activity of SXT while receiving cART than adults [39, 40].

SMX has another benefit as prophylaxis; it is associated with reduced malaria incidence among infants with possible exposure to HIV infection. SMX's protective benefits were more pronounced in asymptomatic infections, potentially affecting the malaria reservoir responsible for transmission. Therefore, SMX prophylaxis may have important individual and public health assets [41]. Although perhaps uncommon, MDR-TB may emerge among TB-infected children and alternative anti-TB drugs may therefore be needed [37]. More investigation on the clinical utility of SXT in the treatment of children infected with MDR-TB is warranted.

Last but not least, the data about the PK/PD target and the pharmacokinetic population model in this thesis are important tools to help explore the efficacy and the optimal dosage of SXT in the treatment of TB while minimizing the toxicity and the risk of resistance during treatment.The pharmacological properties and drug interactions of SXT are already well understood and with the work presented in this thesis, we have made important steps in

our knowledge about the pharmacokinetics, pharmacodynamics and short-term safety of SXT in TB patients. Anti-tubercular activity of SXT against active and latent TB in animal models and humans, and long-term safety in TB patients will be an interesting subject for further investigations.

In conclusion, further evaluations are needed to approve the use of SMX for the treatment of TB. If the efficacy of this drug is established, it will save the lives of many people that currently die every day because of TB in low- and middle-income countries.

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SUMMARY

Treatment of multidrug-resistant tuberculosis (MDR-TB) is challenging because of the high toxicity of second-line drugs and the longer treatment duration compared to drug-susceptible TB. In order to speed up the development of novel treatment for MDR-TB, we suggest considering expanding the indications of already available drugs in the market. In chapter 1 we systematically reviewed *in vitro*, *in vivo* and clinical anti-TB activity of six drugs with antimicrobial activity (phenothiazine, metronidazole, doxycycline, disulfiram, tigecycline and co-trimoxazole), which are not listed in WHO guidelines on MDR-TB treatment but could be potential candidates for evaluation against *M. tuberculosis*. Of the drugs effective against actively replicating TB, co-trimoxazole seems the most promising one because of its consistent pharmacokinetic profile, easy penetration into tissue, safety profile and encouraging *in vitro* activity against *M. tuberculosis*.

Because of these characteristics, we were curious to evaluate the role of sulfamethoxazole, the effective component of co-trimoxazole in TB treatment. Limited data are available in the literature on the pharmacokinetics, pharmacodynamics and safety of sulfamethoxazole in the treatment of MDR-TB. Therefore, we evaluated PK and drug susceptibility along with the tolerability of sulfamethoxazole against *Mycobacterium tuberculosis* in a retrospective study (chapter 2). In this study the ratio of Area Under the free Concentration-time curve (fAUC) from 0 to 24 h relative to the minimal inhibitory concentration ($AUC_{0\text{-}24h}$/MIC) was also evaluated as the best PK/PD parameter to predict the efficacy of sulfamethoxazole. A population pharmacokinetic model was developed showing a consistent PK profile and no significant variability in the area under the curve among the patients studied. The ratio of f $AUC_{0\text{-}24h}$/MIC of sulfamethoxazole exceeded 25 in only one patient. Co-trimoxazole was safe and well tolerated except for one patient who had gastrointestinal side effects after receiving 960 mg of co-trimoxazole.

TB treatment has become increasingly complicated because of co-infection with human immunodeficiency virus (HIV). Therefore, in chapter 3 we measured *in vitro* susceptibility of *M. tuberculosis* to sulfamethoxazole in drug susceptible TB and co-infected HIV/TB patients and compared these results with MICs of sulfamethoxazole from MDR-TB patients in the retrospective study (chapter 2). Because of the comparable susceptibility of *M. tuberculosis* isolates from HIV/TB and MDR-TB patients to sulfamethoxazole, we concluded that Co-trimoxazole is a drug that holds promise for further exploration in the treatment of HIV/TB and MDR-TB patients.

Sulfamethoxazole exposure among HIV/TB co-infected patients appeared highly variable. We therefore decided to explore the relationship between sulfamethoxazole concentration, efficacy, and toxicity. First, we developed a LC–MS/MS analytical method for sulfamethoxazole. This method appeared to provide a reliable and robust quantification of sulfamethoxazole and its toxic metabolite Sulfamethoxazole-*N*-acetyl in serum and

plasma (Chapter 4).

With this novel instrument for analysis (LC–MS/MS), we performed a prospective study in patients with drug-susceptible TB to optimize PK/PD parameters of sulfamethoxazole in the treatment of TB including AUC/MIC and T>MIC (chapter 5). None of these patients who received 960 mg of co-trimoxazole once daily in this study had fAUC$_{0\text{-}24h}$/MIC ratio of sulfamethoxazole greater than 25. The percentage of fT>MIC ranged between 43 and 100% of the dosing interval. We consider these parameters as a starting point to explore the efficacy of sulfamethoxazole and clearly, additional studies are needed to find the precise PK/PD targets and consequently to set the optimal dose of co-trimoxazole for MDR-TB treatment.

In chapter 6, we used dried blood spots (DBS) as an alternative collection procedure to venous blood sampling in order to determine the concentrations of sulfamethoxazole and its metabolites. The stability of these compounds was sufficient to transport DBS cards from highly endemic countries for TB notably, low- and middle income countries around the world to a sophisticated laboratory to perform therapeutic drug monitoring. The calculated AUC$_{0\text{-}24h}$ with DBS samples was comparable to that with venous blood sampling. As even extreme environmental temperatures do not affect the analytical results of sulfamethoxazole and its metabolites during sample collection and transportation, this method is suitable for clinical practice and especially in countries with an extreme climate.

In this thesis we have described the PK and PD parameters of sulfamethoxazole in different groups of TB patients and also the desired characteristics of sulfamethoxazole in different groups of TB patients. In the future in vivo and clinical studies as described in chapter 7 are required to show the precise role of sulfamethoxazole in the treatment of TB.

NEDERLANDSE SAMENVATTING

Behandeling van multidrug-resistente tuberculose (MDR-TB) is een uitdaging vanwege de hoge toxiciteit van tweedelijns geneesmiddelen en de langere duur van de behandeling vergeleken bij geneesmiddel-gevoelige TB. Om het ontwikkelen van nieuwe behandelingen voor MDR-TB te versnellen, stellen wij voor de indicaties van reeds op de markt beschikbare geneesmiddelen uit te breiden. In hoofdstuk 1 hebben we systematisch de *in vitro* (in de reageerbuis), *in vivo* (in diermodellen), en klinische anti-TB-activiteit van 6 geneesmiddelen met antimicrobiële werking (fenothiazine, metronidazol, doxycycline, disulfiram, tigecycline, en co-trimoxazol) beoordeeld. Deze geneesmiddelen komen niet voor in de WHO richtlijn voor MDR-TB behandeling maar moeten worden beschouwd als mogelijke geneesmiddelen tegen *M. tuberculosis*. Van de geneesmiddelen die effectief zijn tegen actief-delende TB bacteriën, lijkt co-trimoxazol de meest veelbelovende te zijn vanwege het voorspelbare geneesmiddelconcentratie, gemakkelijke doordringen in het weefsel, veiligheidsprofiel, en veelbelovende *in vitro* activiteit tegen de TB bacterie (*Mycobacterium (M.) tuberculosis)*.

Vanwege deze eigenschappen wilden wij de rol evalueren van sulfamethoxazol , het actieve bestanddeel van co-trimoxazol in de behandeling van TB. Beperkte data is beschikbaar in de literatuur over de farmacokinetiek, farmacodynamiek , en veiligheid van sulfamethoxazol in de behandeling van MDR-TB. Daarom hebben we geneesmiddelconcentratie en minimaal remmende concentratie samen met de verdraagbaarheid van sulfamethoxazol tegen *de* TB bacterie geëvalueerd in een retrospectieve studie (hoofdstuk 2). In deze studie is de verhouding van het oppervlak onder de concentratie-tijd curve van de niet-eiwit gebonden of vrije fractie (*f*AUC) van 0 tot 24 uur ten opzichte van de minimaal remmende concentratie ($AUC_{0\text{-}24h}$/MIC) geëvalueerd als de beste parameter om de werkzaamheid van sulfamethoxazol te voorspellen. Een populatie farmacokinetisch model werd ontwikkeld. De variabiliteit in de geneesmiddelconcentratie gedurende de gehele dag van de onderzochte patiënten was beperkt. In slechts 1 geval was de verhouding van $fAUC_{0\text{-}24h}$/MIC hoger dan 25. Co-trimoxazol was veilig en werd goed verdragen, behalve bij één patiënt die maag-darm bezwaren had na inname van 960 mg co-trimoxazol.

TB-behandeling is gecompliceerder geworden door co-infectie met humaan immunodeficiëntie virus (HIV). Daarom is in hoofdstuk 3 de *in vitro* gevoeligheid gemeten van *M. tuberculosis* voor sulfamethoxazol in geneesmiddel-gevoelige TB en co-geïnfecteerde HIV/TB patiënten en zijn deze resultaten vergeleken met MIC's van sulfamethoxazol van MDR-TB patiënten uit de retrospectieve studie (hoofdstuk 2). Vanwege de vergelijkbare gevoeligheid van *M. tuberculosis* isolaten van HIV/TB en MDR-TB patiënten voor sulfamethoxazol, hebben we geconcludeerd dat co-trimoxazole een geneesmiddel is dat veelbelovend is voor verder onderzoek in de behandeling van HIV/TB en MDR-TB patiënten.

De sulfamethoxazol bloed concentratie in HIV/TB co-geïnfecteerde patiënten bleek zeer

variabel. Derhalve besloten we de relatie tussen sulfamethoxazol concentratie, effectiviteit en toxiciteit te verkennen. Daartoe ontwikkelden we eerst een gevoelige analysemethode voor sulfamethoxazol met behulp van vloeistof chromatografie en massaspectrometrie (LC-MS/MS). Deze methode bleek een betrouwbare en robuuste kwantificatie van sulfamethoxazol en het toxisch metaboliet n-acetyl-sulfamethoxazol in serum en plasma te verschaffen (hoofdstuk 4).

Met de LC-MS/MS deden we een prospectieve studie in patiënten met geneesmiddel-gevoelige TB voor het vaststellen van de PK/PD parameters van sulfamethoxazol in de behandeling van TB, inclusief AUC/MIC en T>MIC (hoofdstuk 5). Geen van deze patiënten die 960 mg co-trimoxazol eenmaal per dag kregen in deze studie had een $fAUC_{0\text{-}24h}$/MIC verhouding van sulfamethoxazol boven de 25. Het percentage van *f*T>MIC varieerde van 43 tot 100% van het doseringsinterval. We beschouwen deze parameters als startpunt voor het verkennen van de effectiviteit van sulfamethoxazol en het is duidelijk dat er aanvullende onderzoeken nodig zijn om de precieze PK/PD doelen te vinden en vervolgens de optimale dosering van co-trimoxazol bij de behandeling van MDR-TB vast te stellen.

In hoofdstuk 6 hebben we op filterpapier gedroogde monsters van een enkele druppel bloed (DBS) gebruikt; DBS is een alternatief voor uit de ader (veneus) afgenomen bloedmonsters om de concentraties van sulfamethoxazol en haar afbraakproducten (metabolieten) te bepalen. De stabiliteit van deze verbindingen was voldoende om DBS kaarten te ge bruiken voor landen met lage- en middeninkomens waar TB nog veel voorkomt. Deze gedroogde bloedmonsters kunnen makkelijk verstuurd worden naar centrale laboratoria (vaak in de hoofdstad) of naar een internationaal referetie centrum om therapeutische drugmonitoring uit te voeren. De berekende $AUC_{0\text{-}24h}$ van DBS monsters was vergelijkbaar met dat van veneuze bloedmonsters. Omdat zelfs hogetemperaturen geen invloed hebben op de analyseresultaten van sulfamethoxazol en haar metabolieten tijdens monstername en transport, is deze methode geschikt voor de klinische praktijk en vooral in landen met een warm klimaat.

In dit proefschrift hebben wij de PK en PD parameters van sulfamethoxazol in verschillende groepen van TB patiënten beschreven, evenals de gewenste eigenschappen van sulfamethoxazol in de verschillende groepen van TB patiënten. In de toekomst zijn *in vivo* en klinische onderzoeken nodig zoals beschreven in hoofdstuk 7, voor het aantonen van de precieze rol van sulfamethoxazol in de behandeling van TB.

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LIST OF PUBLICATIONS

1. Alsaad N, Dijkstra JA, Akkerman OW, de Lange WC, van Soolingen D, Kosterink JG, van der Werf TS, Alffenaar JW. Pharmacokinetic Evaluation of Sulfamethoxazole at 800 Milligrams Once Daily in the Treatment of Tuberculosis. *Antimicrob Agents Chemother* 2016; 60: 3942-3947.

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